A Stress Response Comparison: LC/MS metabolomics analysis of *Saccharomyces cerevisiae* exposed to the immunosuppressant drugs FK506 and Cyclosporin A

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Introduction

Baker's yeast, Saccharomyces cerevisiae, is extensively used as a model organism for all eukaryotic cells, facilitating research into the biochemistry and biological pathways of more complex but closely related mammalian cells.[1,2,3] Metabolomics represents a quickly growing component in the field of systems biology. It entails the study of an organism's metabolome, or complete set of small molecule metabolites comprised of a wide variety of compounds. A robust controlled extraction protocol is required for studying particular pathways, especially when comparing response to environmental stress.[4,5] We have developed and used an optimized extraction technique. We cultured in parallel, exposing cultures to vehicle control or immunosuppressant, followed by Ca^{2+} exposure, with the goal of perturbing the calcineurin and any other Ca^{2+}/immunsuppresant responsive pathways.[6,7] Sample analysis was done using a liquid chromatograph coupled with a quadrupole/time-of-flight (Q-TOF) mass spectrometer. Data analysis was done using Agilent's MassHunter Qualitative Analysis and MassProfiler Professional software as well as METLIN and AMRT database platforms.

Experimental

S.cerevisiae strain BJ5459 was cultured in parallel and at an OD_{600} of 1.0 exposed to vehicle control (calcium control-CC), 4µg/mL FK506 (FK), or 4µg/mL cyclosporinA (CA) for 1hr. An equal fraction of media containing $CaCl_2$ was added, to a final concentration of 100mM. All cultures were again monitored until an OD_{600} of 1.0 was reached, then centrifuged and washed with phosphate buffered saline (PBS) to remove any residual media. Quenching was done with 1mL of methanol added to the final pellet @ -40° C. After quenching, the final sample was lyophilized. 30mg of dry sample was wet milled using a Retsch MM301 ball mill mixer, in 2 mL Eppendorf tubes in an extraction solvent of 6:3:3 chloroform:methanol:water with a single 5 mm ball bearing used as a tube insert to facilitate mechanical rupture of the yeast. External standards were added prior to milling. Nine samples for each condition were processed for 3 1 min. cycles at 30 Hz. Liquid-liquid extraction (LLE) resulted in polar and non-polar phases. The polar phase supernatants were filtered through 10 kDa ultrafiltration membranes to ensure removal of any residual protein/debris and analyzed via electrospray ionization (ESI) on a Q-TOF MS.

External Standards (ESTDs): External standards were added to monitor reproducibility of metabolite extraction in both ion modes. 1-naphthylamine and 9-anthracene carboxylic acid were added to the milling tubes at a concentration 5 µg/mL prior to milling/extraction.

Experimental

Instrumentation:
An Agilent 1290 Series HPLC system with binary pump and degasser, well plate autosampler with thermostat, thermostatted column compartment, and an Agilent 6530 Accurate-Mass Q-TOF mass spectrometer with an ESI source operated in the positive ion mode were used. Dynamic mass axis calibration was achieved by continuous infusion of a reference mass solution using an isocratic pump connected to a dual nebulizer electrospray ionization source. A 2–98% linear gradient of solvent A (0.2% acetic acid in water) to B (0.2% acetic acid in methanol) was employed over 16 min followed by a solvent B hold of 2 min and a 5 min post-time. An Agilent ZORBAX C18 SB-Aq column 2.1 mm×50 mm, 1.8 µm was used to separate the yeast extracts. An Agilent ZORBAX C-8, 2.1 mm× 30 mm, 3.5 µm guard column was placed in series in front of the analytical column. Autosampler temperature, 4°C; injection volume 5 µL, column temperature 60°C; and flow rate of 0.6 mL/min.

Data Analysis:
-Compounds were extracted from the raw data files using the Molecular Feature Extraction (MFE) algorithm in Agilent MassHunter Qualitative software. This finds all ions that are related, including isotopes and any adducts, such as Na+ or K+ and dimers. Then sums all ion signals into one value (feature). This is fully automated.
-The processed data files were further analyzed using Agilent Mass Profiler Professional (MPP), filtering the MFE results and resulting in a culled list of compounds of interest. The Extracted Ion Chromatogram (EIC) for each of these compounds were evaluated, resulting in a final list of target masses.
-Compound identification was then performed using; 1) METLIN Personal Compound Database (PCD) for LCMS identification 2) METLIN Personal Compound Database and Library (PCDL) for LCMS/MS confirmation 3) Agilent Accurate Mass Retention Time (AMRT) database 4) Molecular Formula Generation (MFG) algorithm*

**LC/MS, METLIN and ms/ms content from Dr. Gary Siuzdak at Scripps Research Institute.*

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Results

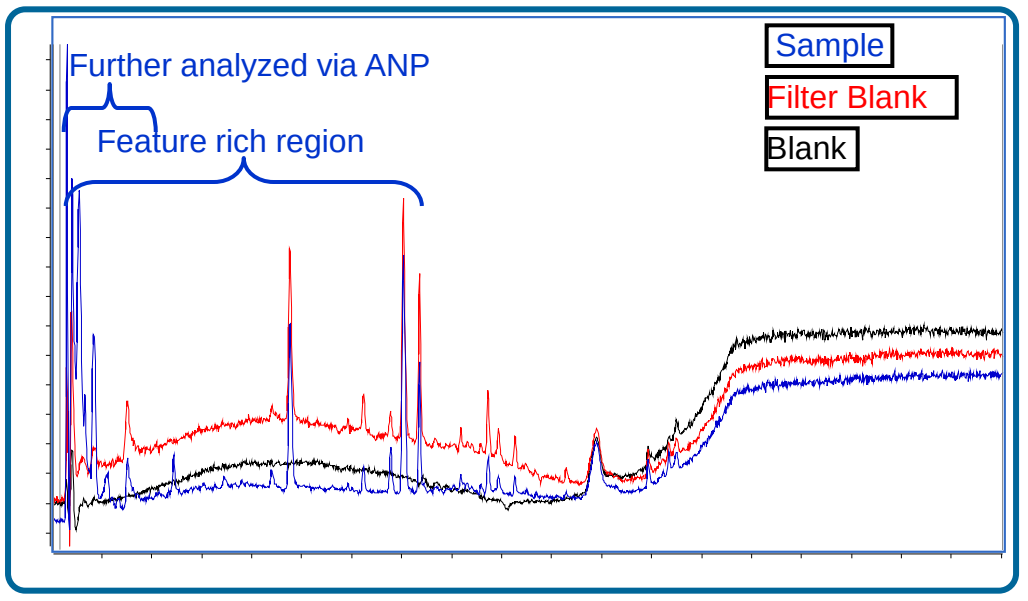


Figure 1: Overlay of TICs detected in ESI pos mode, after separation by reverse phase (RP, C-18 SB-Aq) chromatography. Compounds eluting near the void volume were further separated by Aqueous Normal Phase (ANP) chromatography.

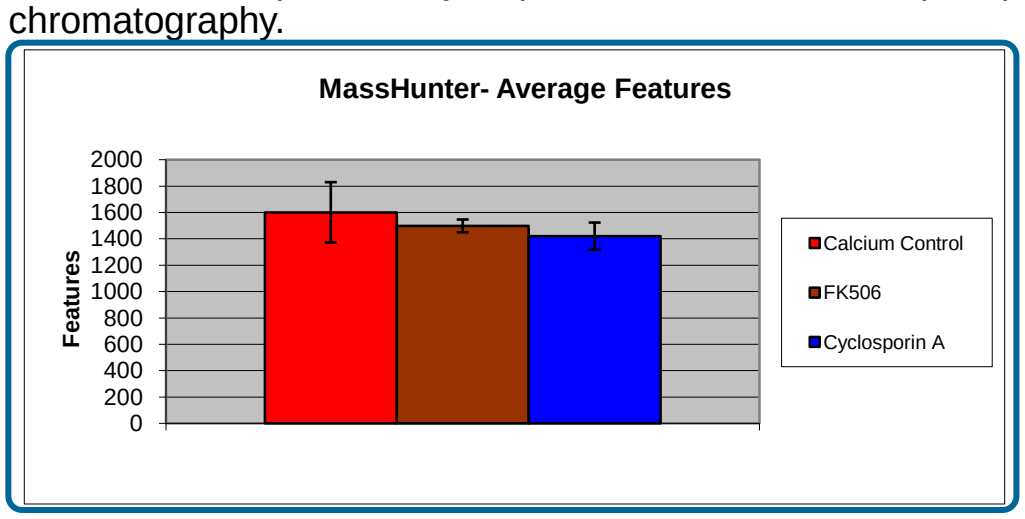


Table 1: The average number of features found by MassHunter Qualitative Analysis for each sample set acquired in ESI pos mode.

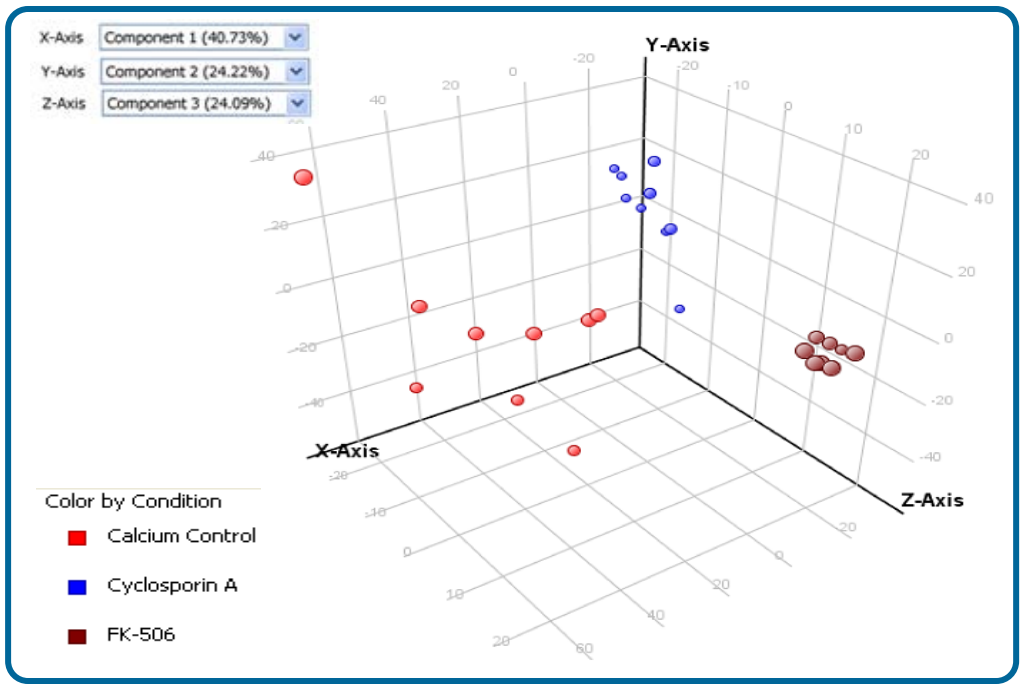


Table 2: Principal component analysis (PCA) plot generated using MPP on each of the 3 culture conditions for ESI pos mode (n=9). Note the tight grouping of FK and CA samples, indicating good reproducibility of biological replicates and separation of the three conditions.

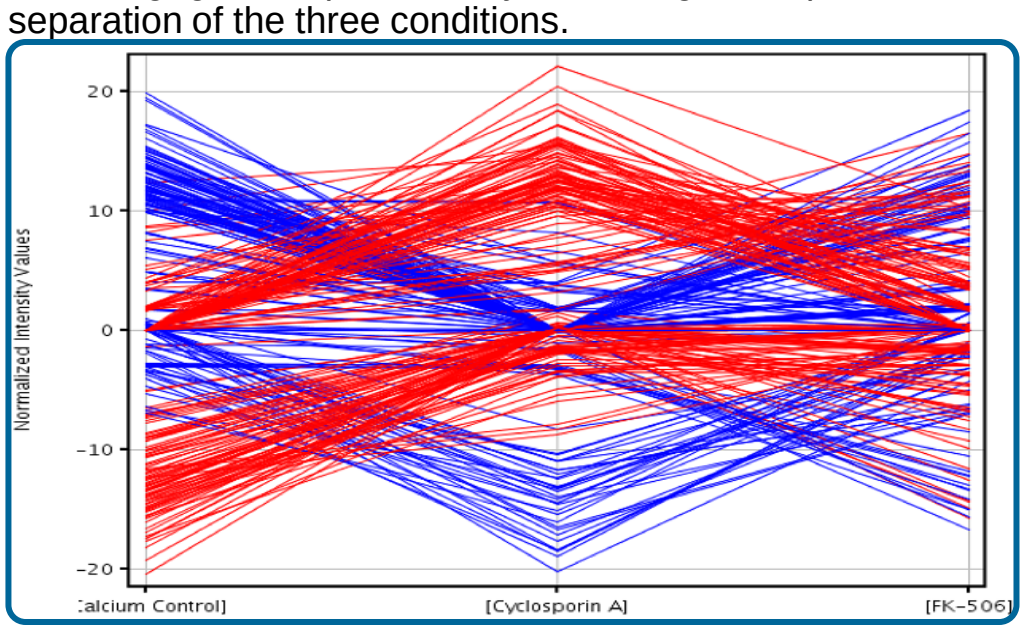
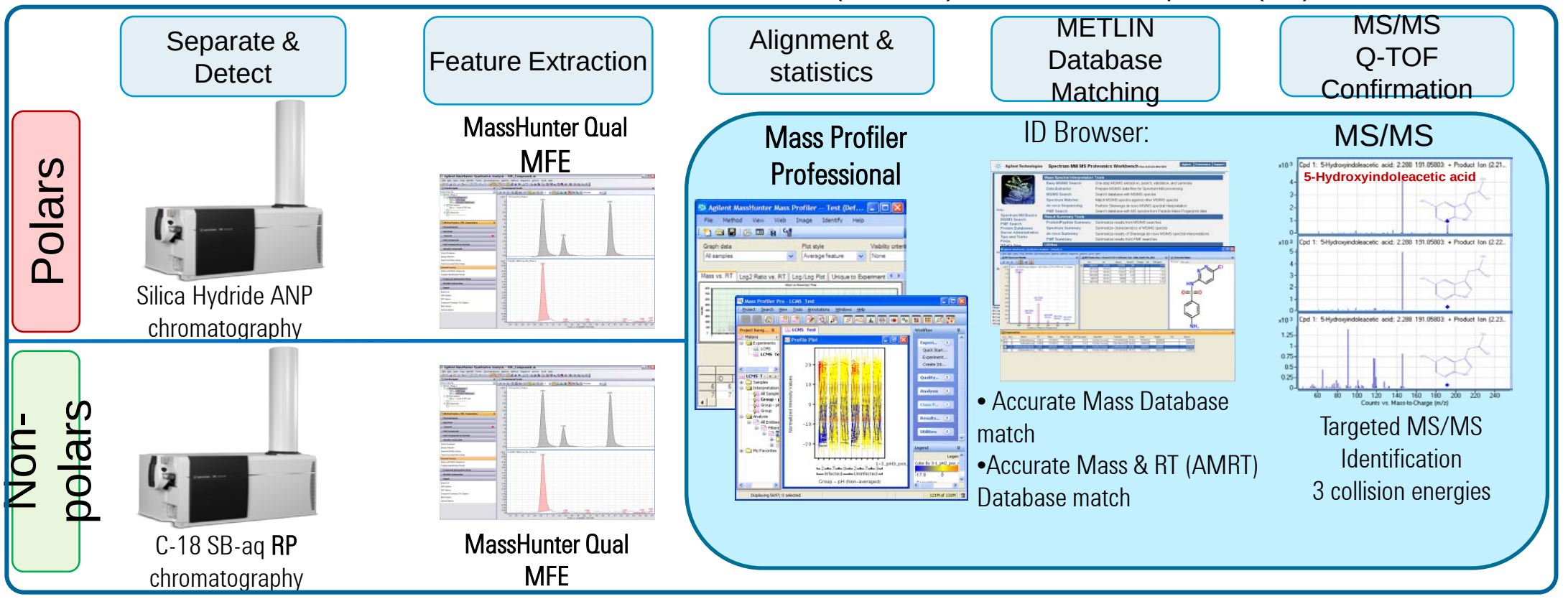


Table 3: Profile plot showing normalized abundance profiles for all 3 culture conditions based on 328 compounds. These were compounds present in 6 out of the nine 9 biological replicates in at least one of the three conditions. Profile groupings show response to immunosuppressant treatment (FK & CA) and/or to Ca^{2+} exposure (CC).

Agilent Metabolomics Workflow



Agilent Technologies

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Results and Discussion

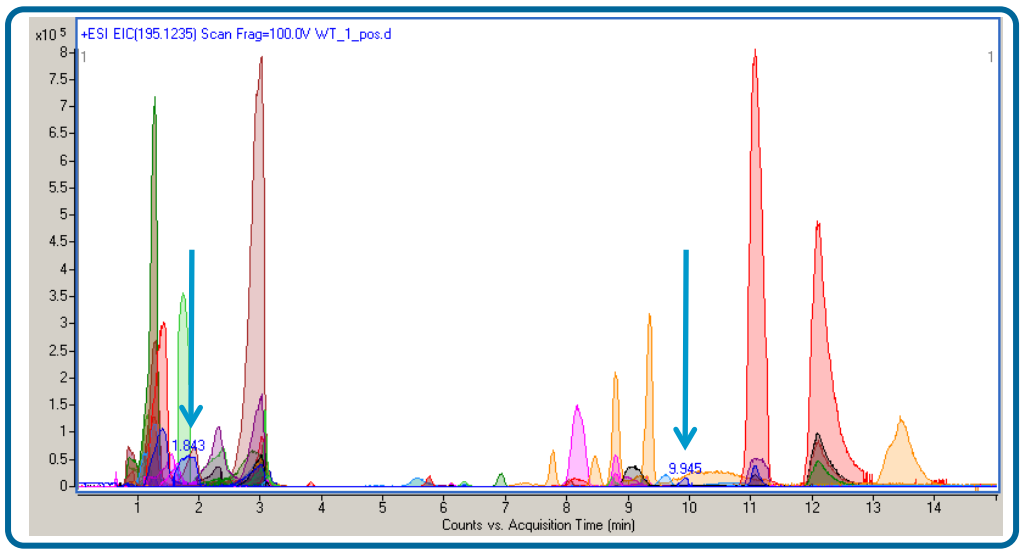

Figure 2: Overlay of EICs for polar compounds collected in ESI pos mode after ANP chromatographic separation on a silica hydride column. The two compounds at RT 1.843 and RT 9.945 minutes (indicated by arrows) were present in the RP TICs (Figure 1) in close proximity to the void volume. These compounds were separated by ANP, with increased retention and therefore more confident database matches.

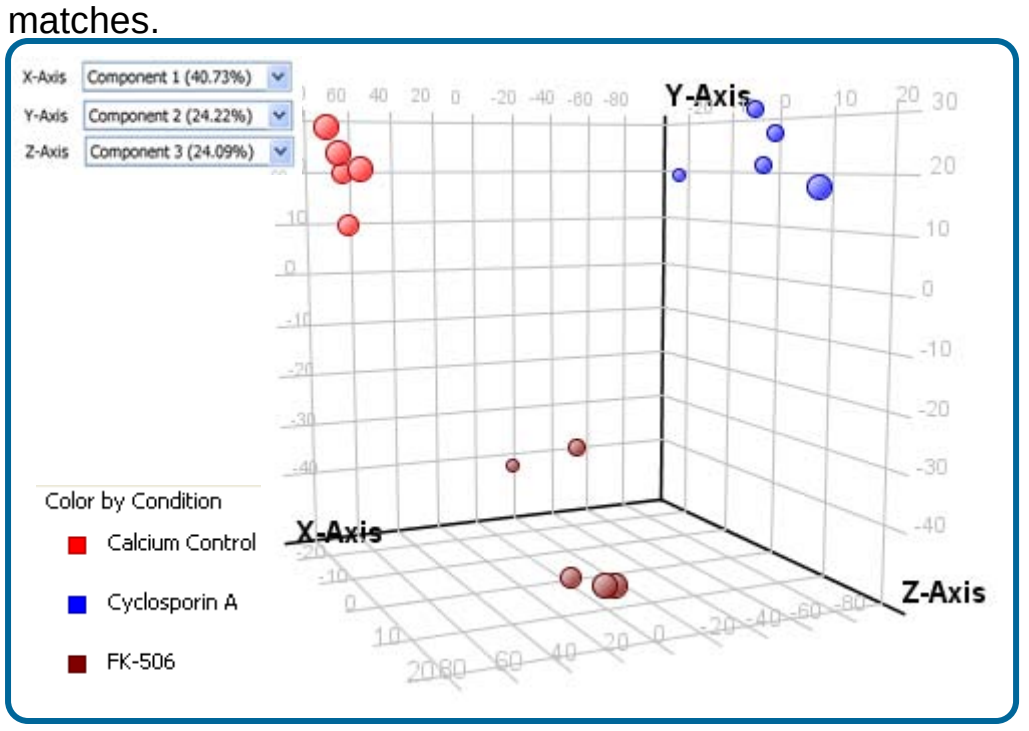

Table 4: Principal component analysis (PCA) plot generated using MPP on each of the 3 culture conditions for ESI pos mode generated using ANP chromatography (n=5). Note the tight grouping of FK and CA samples again indicating good reproducibility of biological replicates and separation of the three conditions.

Compound List

Name	RT	RT (DB)	Mass	Mass (DB)	Formula (DB)	Score (DB)
Riboflavin (Vitamin B2)	4.133	4.34	376.1372	376.1383	C17H20N4O6	100
Riboflavin (Vitamin B2)	4.205	4.34	376.138	376.1383	C17H20N4O6	73.56
Riboflavin (Vitamin B2)	4.205	4.34	376.1383	376.1383	C17H20N4O6	93.76
Riboflavin (Vitamin B2)	4.287	4.34	376.1366	376.1383	C17 H20 N4 O6	60.97
5'-Methylthioadenosine	3.424	3.533	297.0896	297.0896	C11 H15 N5 O3 S	94.35
5'-Methylthioadenosine	3.433	3.533	297.0901	297.0896	C11 H15 N5 O3 S	93.95
5'-Methylthioadenosine	3.435	3.533	297.0902	297.0896	C11 H15 N5 O3 S	94.14

Table 6: Compound identification results obtained directly from the Agilent AMRT Database using targeted ms/ms results. Retention time confirmation for both masses was obtained for all 3 collision energies (10,20,40).

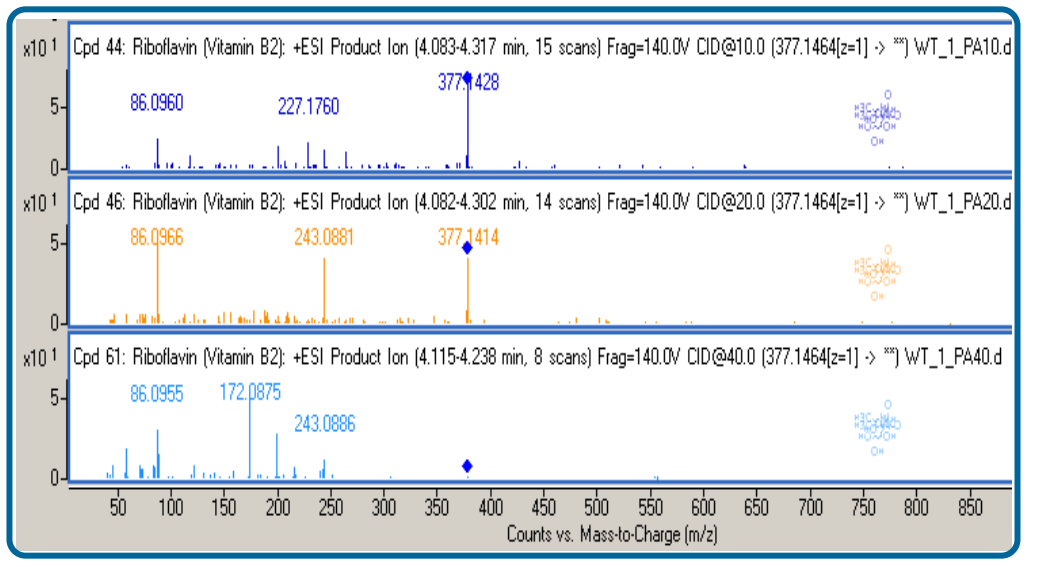

Figure 3: ms/ms results for AMRT confirmed compound Riboflavin, m/z 376.1383 at RT 4.34. At the increased collision energies of 20 and 40, decreased abundance of parent ion is observed in conjunction with higher abundance of lower mass fragments.

Conclusions

- *Novel yeast metabolite extraction protocol yielded a high number of features detected using MFE. Using MPP, many compounds showed reproducible variation between immunosuppressant and calcium stress states.*
- *ANP chromatography was effective in separating many compounds co-eluted near the void volume with RP chromatography.*
- *The Agilent 6530 Accurate-Mass Q-TOF mass spectrometer operated in ESI positive mode with dual chromatographic applications enabled a robust non-targeted characterization of the S.cerevisiae metabolome.*
- *The Agilent Accurate Mass Retention Time database enables orthogonal confirmation of metabolites.*
- *MS/MS analysis will be a useful tool for identifying unknown compounds of interest and making the challenging transition from non-targeted to targeted analysis.*

Acknowledgements

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References

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