Introduction

Yeast is an import model system for biological studies because of its simplicity, ease of manipulation and the availability of strains with individual gene deletions. In this study, a non-targeted maXis impact ultrahigh resolution (UHR)-Q-TOF-based approach was used to identify changes in the metabolism of two arginine biosynthesis pathway deletion mutants (see Figure 1).

Polar metabolites of the arginine biosynthetic pathway are poorly retained in reversed phase C18 chromatography. Hydrophilic interaction chromatography (HILIC) is an option for separating polar metabolites such as amino acids. However, compared to RP chromatography, peaks in HILIC separations are often broader and column re-equilibration requires a longer time. To enable metabolic profiling of ionic and polar metabolites using standard C18 chromatographic separation, the extracted polar metabolites were dansylated according to a protocol published by Guo and Li [1].

This simple derivatization renders target compounds containing primary or secondary amine or phenolic functional groups (see Figure 2A) less polar and leads to increased retention in RP-LC. Additionally, dansylation has been reported to enhance signal intensities in electrospray ionization [1].

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Keywords

<table>
<thead>
<tr>
<th>Instrumentation and Software</th>
<th>Keywords</th>
</tr>
</thead>
<tbody>
<tr>
<td>maXis impact</td>
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<tr>
<td>ionBooster</td>
<td>ionBooster</td>
</tr>
<tr>
<td>Metabolomics</td>
<td>DataAnalysis 4.1</td>
</tr>
<tr>
<td>Structure Elucidation</td>
<td>ProfileAnalysis 2.1</td>
</tr>
<tr>
<td>PCA</td>
<td>SmartFormula3D</td>
</tr>
<tr>
<td>HCA</td>
<td>FragmentExplorer</td>
</tr>
<tr>
<td>Volcano plot</td>
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</tr>
</tbody>
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Figure 1: Simplified schema of yeast arginine biosynthesis highlighting steps blocked in yeast arg7 and arg4 deletion mutants.

Figure 2: (A) Dansylation reaction schema. (B) 2 mDa trace-width high-resolution EICs for dansylated standards reveal a significant retention for derivatized amino acids on a reversed phase column. (C+D) MS and MS/MS spectra of dansylated proline acquired on a maXis impact instrument deliver ultrahigh resolution and mass accuracy, even for low m/z values. (E) SmartFormula3D provides unambiguous elemental composition for precursor and fragment ions. Fragment structures were assigned using the FragmentExplorer, which is based on ChemDraw™ technology.
The FragmentExplorer enabled fast annotation of the MS/MS spectrum with fragment structures. This tool provides an interactive link between SmartFormula3D results, mass spectra and molecular structures and is based on the well-known ChemDraw™ technology for chemical structures. The foundation for this successful interpretation is the excellent mass accuracy and resolution of the maXis impact data, with a resolution of > 22,000 achieved even for low-mass fragment ions (see Figure 2).

Untargeted metabolomics of yeast arginine synthesis pathway deletion mutants

The method described above was applied to study yeast mutants with blockages in the arginine biosynthetic pathway. Figure 1 shows a simplified scheme of the arginine biosynthetic pathway, with the reactions catalyzed by the proteins encoded by \textit{arg4} and \textit{arg7} highlighted.

All features were extracted from the raw data using “Find Molecular Features”. This algorithm can combine isotopic peaks, charge states, and adducts which belong to the same compound into one feature. Based on these extracted features, an unsupervised clustering could clearly separate the derivatized metabolite extracts from wild type and both mutant strains (see dendrogram in Figure 3A). In addition, Principle Component Analysis (PCA) discriminated the three sample types into separate clusters (see scores plot in Figure 3B).

To determine statistically significant differences between sample groups, a student’s t-test was performed comparing wild type vs. \textit{arg7} and wild type vs. \textit{arg4} samples. Alternatively, ProfileAnalysis also allows for ANOVA calculations.

Comparison of wild type vs. \textit{arg7}

Figure 4A shows a screenshot from ProfileAnalysis 2.1. The lower right-hand corner shows the Volcano plot of the t-test result table for the wild type vs. \textit{arg7} comparison, plotting log2 of fold changes vs. -log10 of p-value. The compound with the highest fold change (60-fold increase) in \textit{arg7} samples and a p-value of 0.0000026 had an accurate mass of 408.1588 m/z. The abundance increase for this compound in \textit{arg7} mutants can be readily visualized by plotting EIC traces for all samples within ProfileAnalysis (upper right-hand corner in Figure 4A). The overlay of the measured and simulated spectrum for C\textsubscript{19}H\textsubscript{26}N\textsubscript{3}O\textsubscript{5}S – which was ranked #1 with the best isotopic fit by SmartFormula for the target compound – is shown in Figure 4B. This elemental formula corresponds to dansylated N-acetylornithine. This is in accordance with the assignment of fragment ion structures to the

Experimental

Samples

Wild type yeast and \textit{arg4} and \textit{arg7} gene deletion mutant strains (see Figure 1) were grown on a synthetic growth medium. Six biological replicates were harvested at the same growth phase by centrifugation and washed with PBS buffer. Metabolites were extracted using the Folch method and dansylated as described in [1]. Twenty-one amino acid standards (2.5 \(\mu\text{mol/mL}\) each) were mixed and dansylated, yielding a final concentration of 1 \(\mu\text{mol/mL}\).

UHPLC

U3000 RSLC (Dionex).

Chromatography

Column: Kinetex C18 2.1 x 100mm; 2.6 \(\mu\text{m}\) (Phenomenex).

Column temperature: 30°C. Flow rate: 0.5 mL/min.

Injection volume: 5 \(\mu\text{L}\). Mobile phase: A = H\textsubscript{2}O, B =ACN (each containing 0.1% HCOOH). Gradient: 0.5 min 5% B; linear gradient 5 – 60% B in 20 min; linear gradient 60 – 95% B in 4.5 min; hold 5 min.

MS

maXis impact (UHR-Q-TOF MS, Bruker Daltonics, Inc.).

Ionization: ESI(+) using standard Apollo II ESI or ionBooster source. Scan range: m/z 50–1000. Acquisition rate: 2.5 Hz.

Data processing

SmartFormula3D™ and FragmentExplorer are part of DataAnalysis 4.1 and were used for elemental formula generation and assignment of fragment structures. ProfileAnalysis 2.1 (Bruker Daltonics, Inc.) was used for statistical data evaluation.

Results

Analysis of dansylated amino acids – method development

A mixture of pure amino acids was dansylated (see Figure 2A) and separated by C18 RP chromatography. The high mass accuracy of the acquired full scan MS data enabled creation of 2 mDa trace-width high-resolution Extracted Ion Chromatograms (hrEIC) for the target compounds, providing very high selectivity (Figure 2B). Derivatized standard compounds were retained on the reversed phase column and had very narrow peak widths. As shown for the example of dansyl-proline (Figure 2C), the correct elemental formula could be generated with a mass error of 0.5 ppm and a very good mSigma value. This value provides a measure of the goodness of fit for a measured versus theoretical isotopic pattern. SmartFormula3D correctly assigned the elemental formulas for the observed precursor and fragment ions (see Figure 2E) by taking into account accurate mass and isotopic pattern information within MS and MS/MS spectra.
Table 1: Selected compounds that differed significantly between wild type and arg4 mutant yeast samples.

<table>
<thead>
<tr>
<th>Feature</th>
<th>p-value (t-test)</th>
<th>Fold change (\text{arg4}/\text{wt})</th>
<th>Calculated elemental formula</th>
<th>Tentative ID</th>
</tr>
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<tr>
<td>1</td>
<td>5.1 min : 262.59 m/z (2+)</td>
<td>1.73E-07</td>
<td>(\text{C}<em>{22}\text{H}</em>{31}\text{N}<em>{5}\text{O}</em>{8}\text{S})</td>
<td>Dns-Argininosuccinic acid</td>
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<tr>
<td>2</td>
<td>6.3 min : 367.10 m/z (1+)</td>
<td>0.00006</td>
<td>(\text{C}<em>{16}\text{H}</em>{19}\text{N}<em>{2}\text{O}</em>{6}\text{S})</td>
<td>Dns-Aspartate</td>
</tr>
<tr>
<td>3</td>
<td>7.3 min : 408.16 m/z (1+)</td>
<td>0.00001</td>
<td>(\text{C}<em>{19}\text{H}</em>{26}\text{N}<em>{3}\text{O}</em>{5}\text{S})</td>
<td>Dns-N-Acetylornithine</td>
</tr>
</tbody>
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corresponding high-resolution MS/MS spectrum that was enabled by integrated use of SmartFormula3D and the FragmentExplorer (Figure 5A). N-acetylornithine is the metabolite upstream of the reaction catalyzed by ornithine acetyltransferase, which is absent in arg7 gene deletion yeast mutants (Figure 5B).

**Comparison of wild type vs. arg4**

Table 1 lists three selected compounds which were significantly different (p-value < 0.005) in the comparison of arg4 and wild type samples. Elemental formulas were calculated by SmartFormula using accurate mass and isotopic pattern information. Compounds were tentatively identified based on these molecular formulas and taking retention time information for available standards into account.

An accumulation of argininosuccinic acid and N-acetylornithine would be expected in arg4 mutant yeast cells. A decrease in aspartate was also observed, which could be caused by feedback inhibition triggered by the accumulation of a biosynthetic precursor of arginine biosynthesis.

In addition, this approach revealed previously unknown metabolic changes introduced by the arg4 gene deletion (data not shown). The biochemical implications of these metabolites, which are not directly connected to arginine biosynthesis, are currently being investigated.

**New ionBooster source improves sensitivity for dansylated metabolites**

Guo and Li described an increase of 1–3 orders of magnitude for dansylated compounds compared to the corresponding non-derivatized metabolites in “classical” ESI ionization [1]. Here we wanted to study the effect of using the new ionBooster source to further increase sensitivity for dansylated analytes. The ionBooster enhances sensitivity and lowers detection limits by increasing ionization efficiency. This is achieved by maintaining a controlled vaporizing temperature inside the source to enhance desolvation of analyte ions, even when using high eluent flow rates typical for UHPLC separations. The ionBooster has simple operating parameters and nitrogen consumption requirements similar to those of conventional ion sources.

Figure 6 shows an overlay of base peak chromatograms (BPC) from dansylated amino acid standards separated using the same UHPLC method and ionized using either standard electrospray or ionBooster. Using the ionBooster led to an approximately ten-fold increase in the intensity of dansylated compound peaks. The specific improvement in sensitivity offered by the ionBooster is affected upon the type of compound and its specific chemical characteristics and thermal properties. Here, dansylation seems to make compounds chemically similar, so that sensitivity for detecting an entire class of derivatized metabolites can be increased.

Compared to the classical electrospray ionization used for metabolite profiling in this study, we expect that using the ionBooster will enable detection of even more lower-abundance compounds in yeast arginine mutant strains. In addition, the higher efficiency of precursor ionization will increase sensitivity and facilitate compound identification by increasing fragment ion intensities in MS/MS spectra.

**Figure 5:**
(A) Correlation of tentative structure for C_{19}H_{26}N_{3}O_{5}S (N-acetylornithine) with SmartFormula3D result and MS/MS spectrum. FragmentExplorer links molecular formulas from the SmartFormula3D result with structural suggestions for fragment ions and enables fast annotation of the MS/MS spectrum. (B) N-acetylornithine is the metabolite upstream of the reaction catalyzed by ornithine acetyltransferase, which is absent in arg7 gene deletion yeast mutants.
## Conclusion

Dansylation enables a straightforward and robust reversed phase LC separation of polar primary metabolites like amino acids. This strategy was applied to study metabolic profiles of yeast arginine biosynthetic pathway mutants and revealed an accumulation of precursor metabolites before the blocked enzymatic reactions. This demonstrates the utility of using non-targeted profiling to determine metabolic pathway perturbations in deletion mutant yeast strains.

Data acquired on the maXis impact provided high mass accuracy and resolution across a wide mass range without compromising sensitivity. The combination of unparalleled data quality with SmartFormula3D and FragmentExplorer, which are features of Bruker’s Compass software, permitted unambiguous elemental formula generation and structural confirmation – thereby providing a solution to one of the most difficult challenges in metabolomics research: compound identification.

The ionBooster source increased sensitivity for detecting dansylated metabolites by a factor of ten. In future experiments this will enable determination of even more subtle changes that have not yet been detected by classical electrospray ionization. In addition, the higher intensity will facilitate identification of compounds which were significantly changed in metabolic profiles of mutant strains and are not associated with the arginine biosynthetic pathway.

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### References


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For research use only. Not for use in diagnostic procedures.