High-throughput comprehensive coverage of hydrophilic and hydrophobic metabolites in beer utilizing a dual separation/high resolution accurate mass spectrometry system

Ioanna Ntai[1], Anas Kamleh[2], Martin Samonig[3], Stephanie Samra[1], Aran Paulus[1], Ralf Tautenhahn[1], Amanda Souza[1], Andreas Huhmer[1], [1]Thermo Fisher Scientific, San Jose, CA, USA, [2]Thermo Fisher, Sweden, [3]Thermo Fisher Scientific, Germering, Germany

ABSTRACT

Purpose: Develop a workflow that incorporates HILIC and RP separation for the high-throughput comprehensive coverage of hydrophilic and hydrophobic metabolites in a sample.

Methods: A Thermo Scientific™ Vanquish™ Duo UHPLC system was coupled to a Thermo Scientific™ Q Exactive™ HF mass spectrometer and beer samples were analyzed in both positive and negative mode. Data were processed using Thermo Scientific™ Compound Discoverer™ software suite for unknown identification and differential analysis.

Results: This dual LC/MS system was successfully applied in the differential analysis of beer with increased throughput, without compromising reproducibility or compound detection.

INTRODUCTION

Untargeted metabolomics studies aim to detect, measure, and identify as many metabolites as possible in a sample without bias. However, the diversity in structural, chemical, and physical properties of small molecules, renders complete coverage of the metabolome of a given sample challenging. Diverse separation strategies have been developed to improve metabolome coverage, but unavoidably have introduced bias toward hydrophilic or hydrophobic metabolites. Here, we explored the integration of reverse phase (RP) and hydrophilic interaction chromatography (HILIC) to maximize metabolites detected in a single sample, with high reproducibility and increased throughput. This dual UHPLC separation system was coupled to a Thermo Scientific™ Orbitrap™ mass spectrometer and used for untargeted metabolomic analysis of beer samples, identifying compounds important in discriminating flavor and indicating spoilage.

MATERIALS AND METHODS

Sample Preparation

Sixteen beers from California breweries were purchased from retail stores. For each beer, samples were collected at time of purchase and after storage at room temperature or 4° C for 6 and 18 weeks. Samples were extracted with an excess of cold methanol (4x), containing 4 internal standards (D_8-Valine, D_8-Phenylalanine, D4-succinic acid, $^{13}C_6$-adipic acid). After centrifugation, samples were dried and resuspended in water for RP and 9:1 acetonitrile/water for HILIC.

Test Method(s)

A Thermo Scientific™ Vanquish™ Duo UHPLC system equipped with an autosampler with two injection units, two binary pumps and two column compartments, was coupled to a Thermo Scientific™ Q Exactive™ HF mass spectrometer and data were collected in both positive and negative mode for all samples. For the RP separation, a Thermo Scientific™ Hypersil GOLD™ column (15cm × 2.1mm ID) was used and the mobile phase consisted of solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid). The RP gradient is described in Table 1. For the HILIC separation, a Sequant™ ZIC™-pHILIC column (15cm × 2.1mm ID) was used and the mobile phase consisted of solvent A (water with 10mM ammonium acetate, pH 9.8) and solvent B (acetonitrile). The HiLIC gradient is described in Table 2.

Table 1. RP gradient

Time (min)	% B	Flow rate (mL/min)
0	5	0.3
2	5	0.3
18	95	0.3
28	95	0.3
29	5	0.3
36	5	0.3

Table 2. HILIC gradient

Time (min)	%B	Flow rate (mL/min)
0	90	0.25
15	30	0.25
18	30	0.25
20	90	0.25
36	90	0.25

Data Analysis

Data were processed using Thermo Scientific™ Compound Discoverer software suite for unknown identification and differential analysis.

RESULTS

Independent Fluidic Paths for Increased Throughput

We developed a dual liquid chromatography / mass spectrometry (LC/MS) system that combines RP and HILIC separation and we evaluated it for its reproducibility and separation capabilities of complex samples. Two independent UHPLC pumps were incorporated into the system to allow independent control of the two columns (RP and HILIC). This enabled the use of different solvents, additives and pH ranges and, as a result, a broader metabolite coverage. Having two independent fluidic paths, resulted in a 25% decrease in analysis time and increased the overall throughput of the method. This was accomplished by equilibrating one column, while separation was carried out on the other.

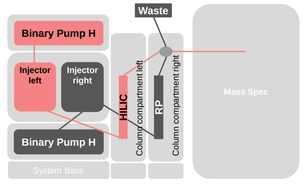


Figure 1. Schematic representation of the Vanquish Duo UHPLC system used in this study illustrating the two separate fluidic paths.

Having two separate flow paths can allow for longer re-equilibration times (Figure 2) or by overlapping the two gradients (Figure 3), the analysis time can be decreased and sample throughput is expedited.

Figure 2. Method gradients for simple column switching without any overlap providing longer re-equilibration times.

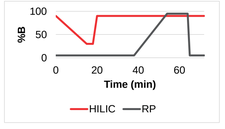
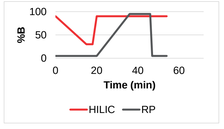


Figure 3. Method gradients for overlap of two separation resulting in a 25% decrease in analysis time and increased throughput.



Increased Throughput Does not Compromise Reproducibility

The increased throughput afforded by this setup did not compromise reproducibility. In figures 4 and 5, the retention times of the internal standards spiked into all samples were monitored over 90 injections, highlighting the robustness of the Vanquish Duo UHPLC system.

Figure 4. The retention times of internal standards remain unchanged during a 90-injection sequence on the HILIC column

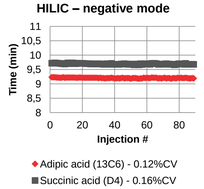
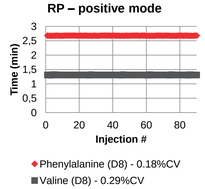


Figure 5. The retention times of internal standards remain unchanged during a 90-injection sequence on the RP column



DIFFERENTIAL ANALYSIS OF BEER SAMPLES

Flavor Discriminants in Different Beer Types

Metabolomic profiles of different beer types were used to detect compounds important in discriminating flavor. Principal component analysis (PCA) of different beer samples showed clear differentiation between IPAs and other beer types.

Figure 6. PCA plot utilizing the HILIC negative mode metabolomic data clearly differentiating IPA and other beer types.

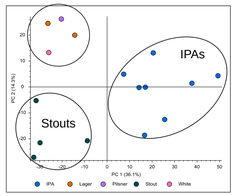
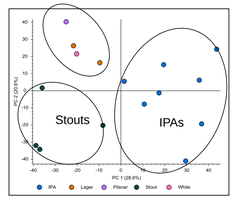


Figure 7. PCA plot utilizing the RP positive mode metabolomic data clearly differentiating IPA and other beer types.



IPAs and stouts have distinguishing metabolomic profiles. Differential analysis within Compound Discoverer 3.0, using volcano and PLS-DA plots, identified some of these discriminating compounds.

Figure 8. Volcano plot utilizing the RP positive mode metabolomic data. Highlighted in blue are the 100 most discriminating compounds between IPA and stout beers. On the left side are the compounds that are lower in IPAs over stouts.

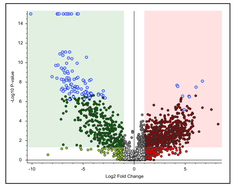


Figure 9. PLS-DA plot utilizing the RP positive mode metabolomic data. Highlighted in blue are the 100 most discriminating compounds between IPA and stout beers.

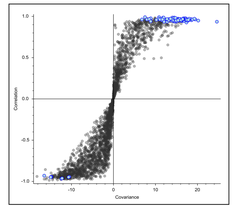
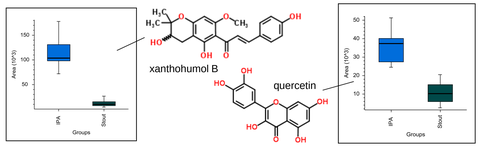


Figure 10. Some of the most discriminating compounds included xanthohumol B and quercetin, phytochemicals known to be derived from the hops during fermentation. These compounds were more abundant in IPAs over stouts as illustrated by the trend chart for xanthohumol B (left) and quercetin (right).

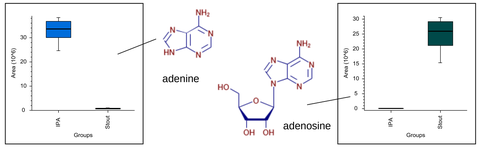


DIFFERENTIAL ANALYSIS OF BEER SAMPLES

Levels Of Purines Are Distinguishing For IPAs Over Stouts

Differing levels of purines and related metabolites were seeing in IPA beers over stouts.

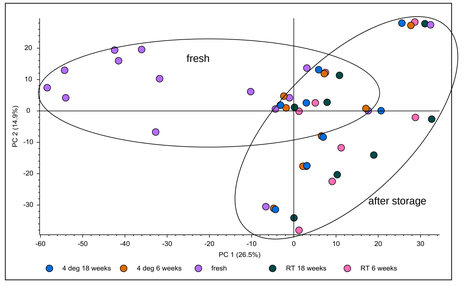
Figure 11. Differences in the levels of purines and related metabolites were detected in IPA and Stout beers as illustrated by the trend chart of adenine (left) and adenosine (right). Interestingly, Adenine is higher in IPAs, but adenosine is higher in Stouts.



Metabolic Profiles of beers change based on temperature and length of storage

Over time metabolite levels change. The biggest change is seeing from time of purchase to 6 weeks. After that, the changes are more subtle.

Figure 12. PCA plot illustrating the metabolic changes that happen during beer storage. Most fresh beers cluster together. The length and temperature of storage after 6 weeks, has minimal effect on the metabolome.



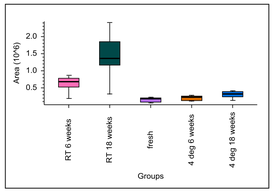
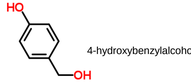


Figure 13. Trend chard for metabolite 4-hydroxybenzylalcohol, a polyphenol associated with hoppy beers. Its intensity increases overtime, especially when the beers are stored at room temperature. It is possibly the byproduct of degradation of larger polyphenols from hops.



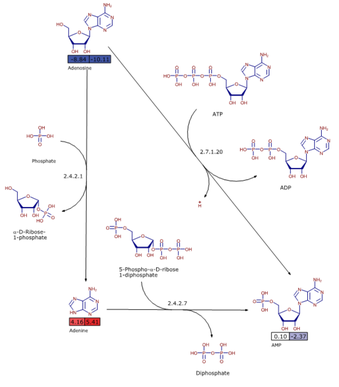
4-hydroxybenzylalcohol

FUNCTIONAL INTERPRETATION OF RESULTS

Overlaying metabolite annotations and differential analysis results on pathways

Biological interpretation of results in any metabolomic study can be performed by overlaying metabolite annotations and differential results on biochemical pathways.

Figure 14. Part of the nucleotide salvage pathway from the Matabolika node of Compound Discoverer 3.0. The log2 fold change for the comparison between IPA and lager and IPA and stout beers are overlaid showing lower levels of adenine and higher levels of adenosine and AMP in IPA over other types of beer.



CONCLUSIONS

- The Vanquish Duo UHPLC system was successfully applied in the differential analysis of beer and provided robust indicators of flavor profiles and degradation. Hop-derived compounds were higher in IPAs. Compounds indicative of degradation were higher with longer storage time at room temperature
- Two independent UHPLC pumps were incorporated into the system to allow independent control of the two columns (RP and HILIC), resulting in a 25% decrease in analysis time and increasing the overall throughput of the method.
- This increased throughput did not compromise reproducibility or compound detection. Internal standards during the analysis exhibited highly reproducible retention times (%CV <1 %)
- One can envision extending application of this system to any metabolomics application, where increased throughput is needed.

TRADEMARKS/LICENSING

PO65285-EN 0518S

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