Single-Injection Method for Simultaneous Quantitation of 30 Metabolites by UHPLC-HRMS

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Overview

Objectives:

- Develop a combined quantitative and qualitative LC-MS/MS workflow utilizing IROA standards
- Apply proposed new workflow to a clinical study for simultaneous quantitation and untargeted metabolomics.

Methods:

A calibration curve composed of 5% U-^{13}C IROA labeled yeast extract (*Saccharomyces cerevisiae*) was paired with 95% U-^{13}C labeled IROA yeast extract as an IS. All samples were spiked accordingly with the 95% U-13C IS. The metabolites of interest within the 5% U-13C yeast and biological samples were validated against standards to confirm *m/z* and concentrations of each analyte. Chromatographic separation was achieved using reverse-phase and hydrophilic interaction chromatography. Quantification was performed by extracting a 5 ppm window around the precursor *m/z* of each analyte with a 30 second chromatographic tolerance and in addition, the MS/MS product ions were used to evaluate differences in quantification between precursor and product ions.

Introduction

Traditional LC-MS metabolomic workflows for biomarker detection typically rely on untargeted global analysis of metabolites with secondary validation of significant biomarkers using a separate targeted assay to quantify and improve measurement reliability. Under this workflow, additional targeted analyses are often limited to a few metabolites due to the restricted availability of standards and the use of additional sample material. Here, we describe the generation of isotopically-labeled yeast extracts for the identification and quantitation of thirty small metabolites in serum/plasma. The 5%- or 95% U-^{13}C extracts were used to generate calibration curves and complex internal standards (IS), respectively, to locate, validate, and quantify biochemical equivalents in plasma samples of subjects with the *CDKN1C* gain-of-function mutation expressing insulin inefficiency.

Instrumentation

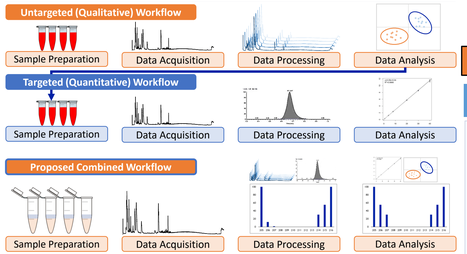
Liquid Chromatography

Method	Reverse Phase	Hydrophilic Interaction
Column	ACE Excel 2 C18 PFP (2.1 x 50 mm, 1.8 µm particle size)	Waters Acquity UPLC BEH Amide (2.1 x 150 mm, 1.7 µm particle size)
Solvent A	0.1% Formic acid in water	20 mM Ammonium Formate in water Adjusted pH to 4 with acetic acid
Solvent B	Acetonitrile	0.1% Acetic Acid in acetonitrile
Flow Rate	350 µL/min	500 µL/min
Gradient A:B	100:0 → 50:50 18 min run with multistep gradient	5:95 → 50:50 20 min run with multistep gradient

Mass Spectrometry

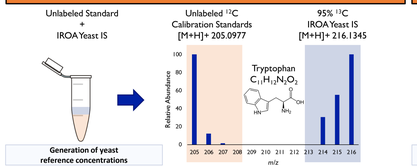
	Thermo Scientific Q Exactive		Sciex x500R QTOF
Source	Ion Max Source with HESI II Probe	**Source**	Turbo V ion source with Twin Sprayer ESI Probe
Scan Range	*m/z* 70-1000	**Scan Range**	*m/z* 50-1000
Resolution	35,000 @ m/z 200	**Resolution**	42,000 @ m/z 956
Capillary Temp.	325° C	**Probe Temp.**	450° C
Spray Voltage	3,500 V	**Spray Voltage**	4,500 V

Metabolomic Workflow

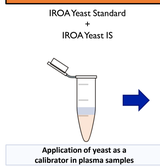


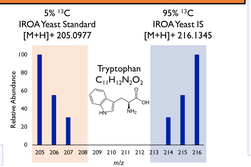
Methods

Quantitation of Yeast Metabolites



Quantitation of Plasma Metabolites





Results and Discussion

IROA as an Injection Standard

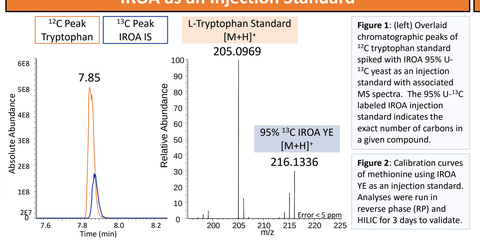
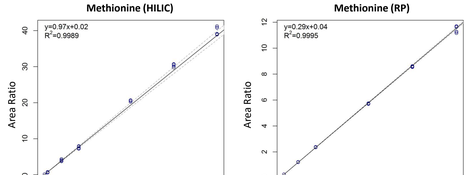
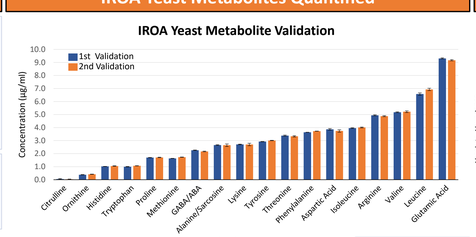


Figure 1: (left) Overlaid chromatographic peaks of ^{12}C tryptophan standard spiked with IROA 95% U-^{13}C yeast as an injection standard with associated MS spectra. The 95% U-^{13}C labeled IROA injection standard indicates the exact number of carbons in a given compound.

Figure 2: Calibration curves of methionine using IROA YE as an injection standard. Analyses were run in reverse phase (RP) and HILIC for 3 days to validate.



IROA Yeast Metabolites Quantified



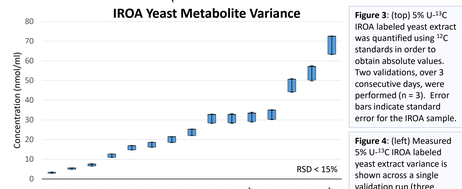


Figure 3: (top) 5% U-^{13}C IROA labeled yeast extract was quantified using ^{12}C standards in order to obtain absolute values. Two validations, over 3 consecutive days, were performed (n = 3). Error bars indicate standard error for the IROA sample.

Figure 4: (left) Measured 5% U-^{13}C IROA labeled yeast extract variance is shown across a single validation run (three consecutive days) with 6 replicates over 3 different concentrations.

IROA as an Internal Standard

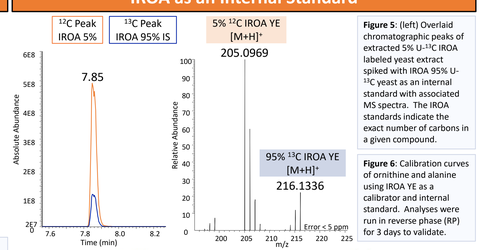
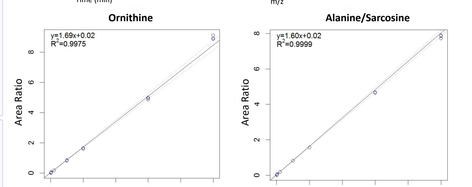


Figure 5: (left) Overlaid chromatographic peaks of extracted 5% U-^{13}C IROA labeled yeast extract spiked with IROA 95% U-^{13}C yeast as an internal standard with associated MS spectra. The IROA standards indicate the exact number of carbons in a given compound.

Figure 6: Calibration curves of ornithine and alanine using IROA YE as a calibrator and internal standard. Analyses were run in reverse phase (RP) for 3 days to validate.



Application of IROA for Absolute Quantitation and Qualitative Analysis

Validation of Quantitation Method

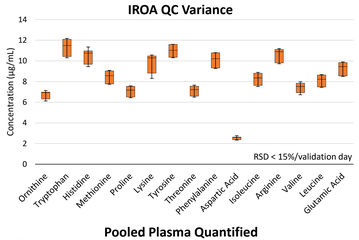


Figure 7: Boxplots of the quality control (QC) standards analyzed during the validation of the quantitation method (n = 3). Validation of the method was performed over the course of 3 consecutive days with fresh extracted samples. QCs were composed of 30 ^{12}C standards extracted with the 95% U-^{13}C labeled yeast extract as an internal standard. QC data was accepted based on an RSD of < 15% per validation day. Processing of QC data were performed on Thermo Scientific Xcalibur and Sciex OS.

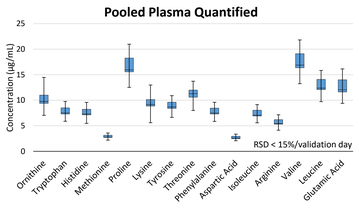


Figure 8: Pooled plasma obtained from the American Red Cross were used as QC samples during the validation of the quantitation method. The plasma was extracted by protein precipitation method using acetonitrile: methanol: acetone (8:1:1, v/v/v) and spiked with 95% U-^{13}C labeled yeast extract as an internal standard. Processing of data were performed on Thermo Scientific Xcalibur and Sciex OS.

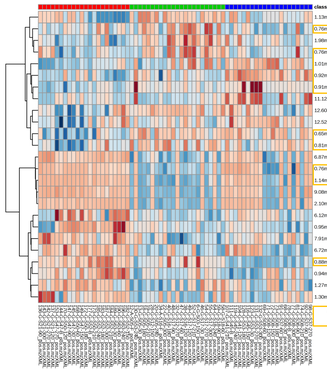
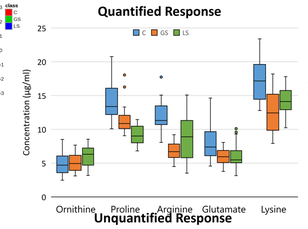
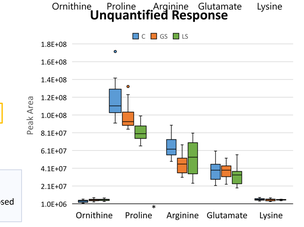


Figure 9: Statistical analysis of human serum samples from two populations with CDK mutations associated with Type 1 Diabetes. The heatmap shows the top 25 features from the identified compounds list. Of the 25 features shown, 10 compounds are able to be quantified by the proposed method. Statistical analysis was performed on MetaboAnalyst 4.0.





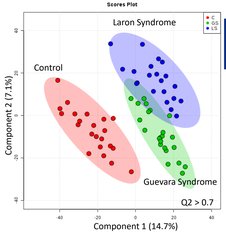


Figure 10: (left) Boxplots showing the response from metabolites using the quantitation method (*top*) compared to the same response from the qualitative data (*bottom*). The quantified response was measured by using the 5% U-^{13}C labeled yeast extract as a calibrator and the 95% U-^{13}C labeled yeast extract as an internal standard. The qualitative data was measured using the ^{12}C peak area of the extracted samples.

Figure 10: (top) PLS-DA of the global metabolomics data. Subjects from the Laron syndrome (LS) cohort and the Guevara syndrome (GS) were compared against control samples. Statistical analysis was performed on MetaboAnalyst 4.0. Data processing was performed using MZmine 2.

Conclusions

- Quantitation results show strong linearity for the analyses of several metabolites including amino acids and their derivatives.
- Quantitation of 5% U-^{13}C IROA yeast metabolites using reverse phase chromatography can be corroborated with HILIC chromatography.
- Quantitation of amino acids using 5% and 95% U-^{13}C IROA yeast in tandem for calibration and as a complex IS is sufficiently stable to generate reproducible results.
- As a tandem workflow, the proposed combined quantitative and qualitative method was successful in obtaining absolute concentrations of several metabolites.
- The untargeted analysis of the CDK mutation samples were sufficiently separated by the three populations sampled from.

Future Directions

Future work will include further analysis of clinical samples using HILIC chromatography. This additional analysis will provide confirmation and better visualization of polar metabolites that are difficult to retain in traditional reverse phase chromatography. For quantitation, this will also corroborate values obtained. Additionally, identifying a reverse phase method optimized for plasma would increase the probability of identifying more precise quantitation. This includes optimizing the mobile phase solvents, solvent for reconstitution of extracted samples, and columns specific for plasma quantitation and qualitative analysis.

References

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