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Isotopologue ratio normalization for non-targeted metabolomics☆

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ABSTRACT

Robust quantification of analytes is a prerequisite for meaningful metabolomics experiments. In non-targeted metabolomics it is still hard to compare measurements across multiple batches or instruments. For targeted analyses isotope dilution mass spectrometry is used to provide a robust normalization reference.

Here, we present an approach that allows for the automated semi-quantification of metabolites relative to a fully stable isotope-labeled metabolite extract. Unlike many previous approaches, we include both identified and unidentified compounds in the data analysis. The internal standards are detected in an automated manner using the non-targeted tracer fate detection algorithm. The ratios of the light and heavy form of these compounds serve as a robust measure to compare metabolite levels across different mass spectrometric platforms. As opposed to other methods which require high resolution mass spectrometers, our methodology works with low resolution mass spectrometers as commonly used in gas chromatography electron impact mass spectrometry (GC–EI-MS)-based metabolomics.

We demonstrate the validity of our method by analyzing compound levels in different samples and show that it outperforms conventional normalization approaches in terms of intra- and inter-instrument reproducibility. We show that a labeled yeast metabolite extract can also serve as a reference for mammalian metabolite extracts where complete stable isotope labeling is hard to achieve.

1. Introduction

Metabolomics, the attempt to measure the levels of all metabolites of a given system under the given conditions, has become increasingly important in biomedical research [1,2]. Metabolomics data can be the basis for biomarker discoveries [3], biotechnological applications, or metabolic flux analysis [4–7].

However, analytical variance poses problems to the comparison of measurements from different runs or instruments, especially in non-targeted metabolomics. Common data treatments like total ion current normalization cannot be used for cross-platform comparisons and only account for certain types of errors like fluctuations in overall sensitivity. Often these techniques are limited to a set of very similar metabolite profiles. Normalization on pool samples can be performed, but this does not take into account the potentially different metabolite profiles with different matrix effects.

Analytical variance is best addressed by adding stable isotope-enriched internal standards to the sample. The addition of stable isotope-enriched compounds to a sample before mass spectrometric analysis is referred to as isotope dilution mass spectrometry (IDMS). IDMS is commonly used for targeted quantitative metabolomics. In non-targeted metabolomics many compounds remain unidentified and can, thus, not be included in any standard mixture. However, this shortcoming can be circumvented by using fully labeled metabolite extracts of a similar sample as reference. For example, metabolite extracts of fully ^{13}C-enriched yeast, bacteria, plant, algae, and filamentous fungi have been used successfully as complex standard mixtures for large scale metabolite quantification or determination of sum formulas [8–13]. So far, they have not been used for automated non-targeted metabolomics.

For liquid chromatography electrospray ionization high resolution mass spectrometry (LC–ESI–HRMS) data, there are methods for non-targeted IDMS available for both semi-quantification and identification of analytes. Bueschl et al. [13] applied complete isotopic enrichment, whereas the isotopic ratio outlier analysis (IROA) [14] uses partial stable isotopic enrichment. Pairs of labeled and unlabeled compounds are automatically detected from the typical isotopic peak patterns. However, these methods are not applicable for low resolution mass spectrometers and hard ionization techniques like electron ionization (EI) which produce a large

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number of fragment ions. Without accurate mass measurements, mass spectral peak patterns arising from fragmentation often cannot clearly be distinguished from isotopic peak patterns. Therefore, other means are necessary for the automated and non-targeted detection of stable isotope-labeled compounds in such data.

Here, we present an approach for GC–EI-MS metabolomics that allows for the robust normalization or semi-quantification of both identified and unidentified metabolites relative to a spiked-in stable isotope-labeled metabolite extract. We used a similar approach as Wu et al. [9] who applied fully ^{13}C-labeled yeast metabolite extract as internal standard. However, their analysis has been very targeted and did not make use of the information on unidentified analytes. We overcome this limitation by employing the non-targeted tracer fate detection (NTFD) algorithm [15] to detect all isotopically enriched compounds within a reference mixture in an automated manner. The intensity ratios of native compounds and the corresponding references are then used to normalize analyte levels in the sample of interest. Additionally, the number of carbon and nitrogen atoms of the unidentified compounds can be obtained. Using this experimental setup, absolute quantification of identified compounds is possible as shown by others [9]. We demonstrate the validity of our methodology by comparing intra- and inter-instrument variation to conventional methods.

2. Materials and methods

2.1. Materials

Chemicals were purchased from Sigma–Aldrich, unless indicated differently. All solvents used were of grade *Chromasolv* or better.

2.2. Culture conditions

To produce the fully labeled reference mixture, *Saccharomyces cerevisiae* strain S90 mating type α was grown on YPD agar at 30 °C for 48 h. A single colony was transferred to 5 mL of liquid YPD medium for an overnight culture, and then to YNB medium containing [$^{15}N_2$]ammonium sulfate and D-[U-^{13}C]glucose (Cambridge Isotope Laboratories, 99% isotopic purity) as sole nitrogen and carbon source again over night. Cultures were incubated on a rotary shaker (Infors Multitron) at 30 °C and 200 rpm. Following another 5 mL YNB labeling culture over night, culture volume was increased to 100 mL. Cultures were inoculated at OD_{600} = 0.1, cell growth was monitored using a cell density meter (Biowave CO8000) and metabolites were extracted in mid-exponential growth phase.

S. cerevisiae strain YJM789 was grown on YPD agar at 30 °C for 48 h. After an over night culture in 5 mL liquid YPD medium, a 10 mL YPD culture was prepared and extracted in mid-exponential growth phase.

A549 cells (ATCC CCL-185) were grown in multi-well plates in DMEM medium (Invitrogen) supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin in an incubator (Sanyo) at 21% O_2, 5% CO_2 at 37 °C.

2.3. Metabolite extraction and standard addition

The yeast culture was centrifuged at 3900 × *g* for 3 min at −10 °C, the pellet resuspended in 2 mL extraction fluid (50%, v/v, methanol in water, −20 °C) and transferred to a reaction tube, prefilled with 600 mg acid-washed glass beads (ø150–212 μm, Sigma–Aldrich). 10 mL of the YPD and 25 mL of the YNB culture were harvested at $OD_{600} \approx 2$. Cell lysis was performed using a Precellys24 (Bertin) homogenizer, equipped with a Cryolys cooling option held at 0 °C, and the following program: 2 × 30 s at 6800 rpm with 30 s pause in-between. After adding 500 μL chloroform, thorough mixing, and centrifugation at 14,000 × *g* for 5 min at 4 °C, the upper aqueous phase was used for analysis of polar metabolites. The labeled polar metabolite extract was diluted 1:10 in methanol:water (1:1, v:v) and stored at −80 °C until use. The interphase forming during the extraction was hydrolysed in 1.5 mL of 6N hydrochloric acid at 99 °C over night. The supernatant was evaporated and the residue was extracted with 1.5 mL methanol:water (1:1, v:v) and diluted 1:10 with methanol:water (1:1, v:v).

To generate the library of labeled compounds 30 μL of unlabeled metabolite extract and 4 μL of the unlabeled hydrolysate were measured separately, and in mixture with 30 μL and 8 μL of $^{13}C^{15}N$-labeled polar extract and interphase.

As internal standards for the yeast YJM789 samples 6 μL of $^{13}C^{15}N$-labeled yeast S90 polar extract and 10 μL interphase hydrolysate were spiked into 100 μL of the polar extract of interest.

A549 cell extract was prepared from 4×10^5 cells. Cells were washed with 1 mL 0.9% (w/v) NaCl and quenched with 400 μL methanol (−20 °C). After adding 400 μL water (4 °C), the cells were scraped off with a cell scraper and the cell suspension was transferred into an Eppendorf tube containing 400 μL chloroform at −20 °C. Tubes were shaken for 20 min at 1400 rpm and 4 °C and centrifuged for 5 min at 16,100 × *g* at 4 °C. A detailed protocol is available in [16]. To 300 μL of the aqueous phase, 6 μL of uniformly $^{13}C^{15}N$-labeled S90 polar extract and 10 μL of interphase hydrolysate were added.

2.4. Sample preparation & GC–MS measurement

The metabolite extracts were transferred to glass vials with micro inserts and dried in a CentriVap vacuum evaporator (Labconco) at −4 °C. Automated sample derivatization was performed by using a multi-purpose sampler (GERSTEL). Dried samples were dissolved in 15 μL pyridine, containing 20 mg/mL methoxyamine hydrochloride and incubated at 40 °C for 60 min under shaking. In a second step, 15 μL *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) were added to the samples and they were further incubated at 40 °C for 30 min under continuous shaking.

GC–MS analysis was performed on an Agilent 7890A GC coupled to an Agilent 5975C inert XL Mass Selective Detector (Agilent Technologies). A sample volume of 1 μL was injected into a split/splitless inlet, operating in splitless mode at 270 °C. The gas chromatograph was equipped with a 30 m DB-35MS capillary column with a 5 m DuraGuard capillary in front of the analytical column (Agilent J&W GC Column).

Helium was used as carrier gas with a constant flow rate of 1.0 ml/min. The GC oven temperature was held at 80 °C for 6 min and increased to 300 °C at 6 °C/min. After 10 min, the temperature was increased at a rate of 10 °C/min to 325 °C and held for 4 min. The total run time was 59.167 min.

The transfer line temperature was set to 280 °C. The MS was operating under electron ionization at 70 eV. The MS source was held at 230 °C and the quadrupole at 150 °C. Full scan mass spectra were acquired from *m/z* 70 to *m/z* 800.

For inter-instrument comparison the samples were also measured on an Agilent 7890B gas chromatograph coupled to an Agilent 5977A mass spectrometer using the same column type and temperature program.

2.5. Chromatogram preprocessing

Deconvolution of mass spectra, peak picking, integration, and retention index calibration were performed using the MetaboliteDetector software [17]. Compounds were identified using an in-house mass spectra library. The following deconvolution settings were applied: Peak threshold: 5; Minimum peak height: 5; Bins per scan: 10; Deconvolution width: 5 scans; No baseline