

# Measurement of isotopologue ratios for metabolic flux analysis using parallel, quasi-simultaneous EI&CI-TOFMS detection with a flexible CI source

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## INTRODUCTION

Metabolic flux analysis (MFA) is an essential tool for understanding cellular metabolism and its regulation. The MFA approach involves introducing <sup>13</sup>C-labeled substrates, such as glucose, into a biological system, tracing of isotopically labeled metabolites within the metabolomic pathways followed by quantifying the isotopologue distributions.

By analyzing these isotopologue distribution of key metabolites, one can gain detailed insights into the activity and regulation of central metabolic pathways, including PPP, glycolysis, and TCA (tricarboxylic acid) cycle. This approach is crucial for understanding cellular metabolism in various contexts.

Accurate isotopologue ratio measurements are critical for reliable flux estimations, but conventional GC-MS methods face challenges due to fragmentation and limited sensitivity. In particular, electron ionization (EI) suffers from a lack of intact molecular carbon backbone information due to strong fragmentation, which complicates isotopologue analysis.

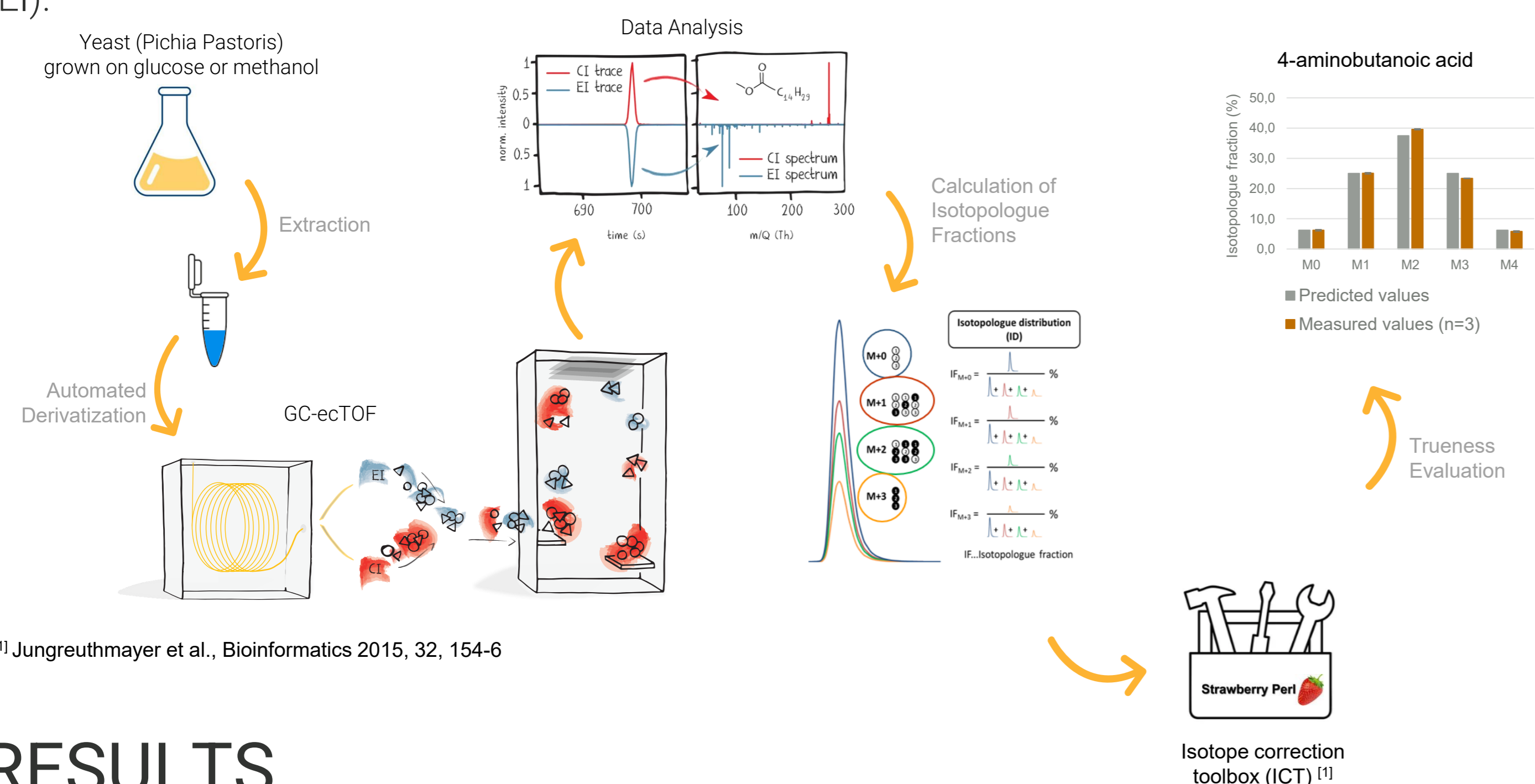
To address these limitations, we present a novel dual ionization GC-EI&CI-TOFMS platform equipped with a newly developed chemical ionization (CI) source. This system enables complementary EI and CI measurements, improving both fragment ion coverage and molecular ion detection. The combined approach enhances isotopologue quantification accuracy and robustness, offering new opportunities for MFA in complex biological systems.

## METHODS

Isotopologue abundances of central carbon metabolites were measured in standard mixtures and yeast cell extracts which grew on natural glucose and 50:50 <sup>13</sup>C labeled methanol.

A validated in-house method with an automated two-step derivatization was applied to test chemical ionization reagent gases employing the benefits of automated reagent gases switching included in the novel GC-TOF system (ecTOF, Bruker Daltonics GmbH, Bremen Germany).

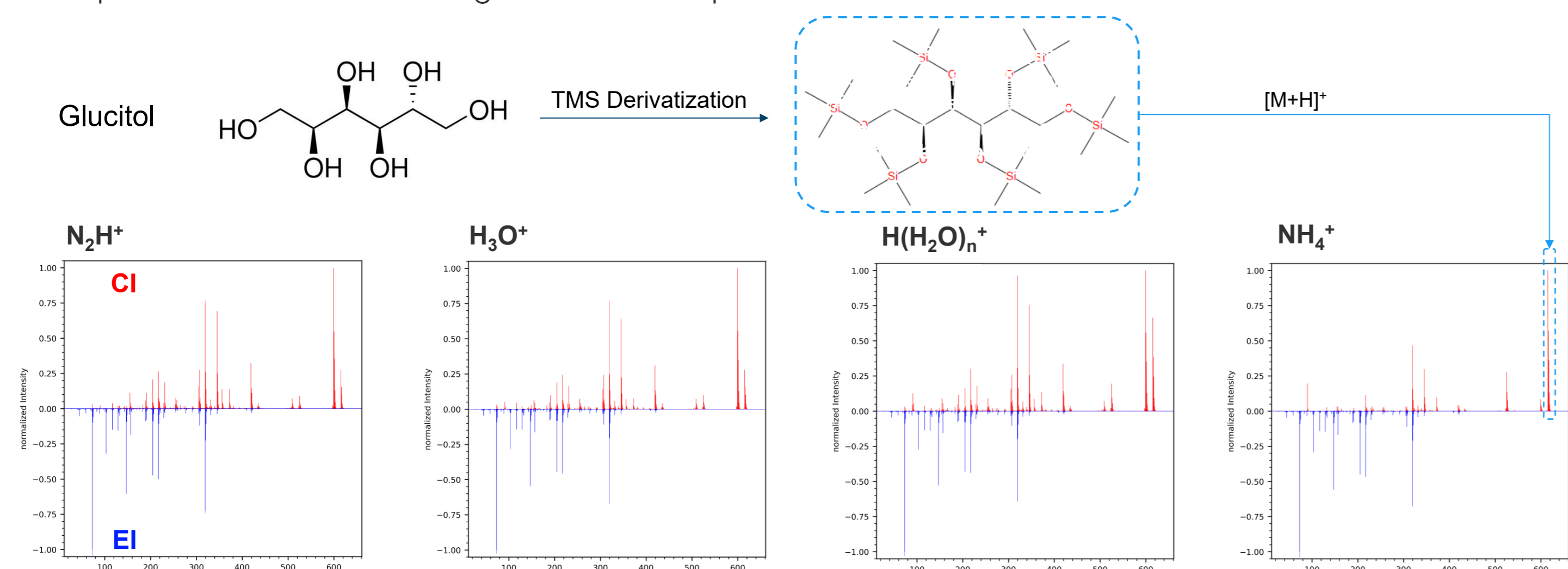
The measured isotopologue intensities were corrected for the contribution of natural abundant isotopes including those added by derivatization. As reference materials are still lacking, trueness of the isotopologue ratios was assessed using an in vivo labeled material of defined isotopologue distributions (ethanolic cell extract of yeast cultivated on 50:50 <sup>13</sup>C labeled methanol). The results of the GC-EI&CI-TOFMS were evaluated in terms of trueness and precision. The combination of EI & CI data was used to extend the targeted list of metabolites. Retrospective untargeted analysis enabled compound identification at a high level of confidence by combining the information on molecular weight (CI) and fragmentation patterns (EI).



## RESULTS

### Choice of CI Reactant – Coverage is Key

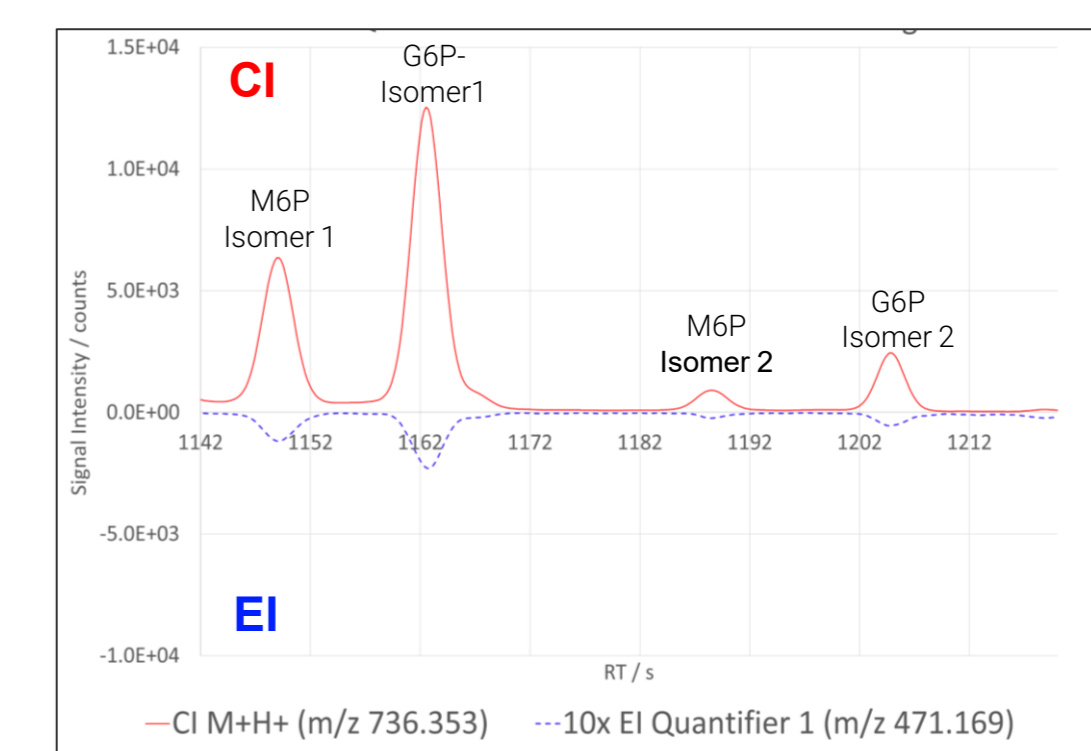
The ecTOF enables automated switching between four default CI reagents within a sequence without requiring hardware modifications. A broad range of reagent selectivity, facilitated by heated permeation tubes, allows for optimized ionization conditions for a wide range of compound classes including derivatized species.



Example Mass Spectra of TMS derivatized glucitol using different CI reactants (left to right:  $N_2H^+$ ,  $H_3O^+$ ,  $H(H_2O)_n^+$  and  $NH_4^+$ ). While the EI spectrum is showing the same spectrum and same intensities, the CI mass spectrum shows increasing  $M+H^+$  signal intensities (absolute increase  $N_2H^+ \rightarrow NH_4^+$  factor x10) and changing fragmentation pattern due to changing excess energy in the ionization process.

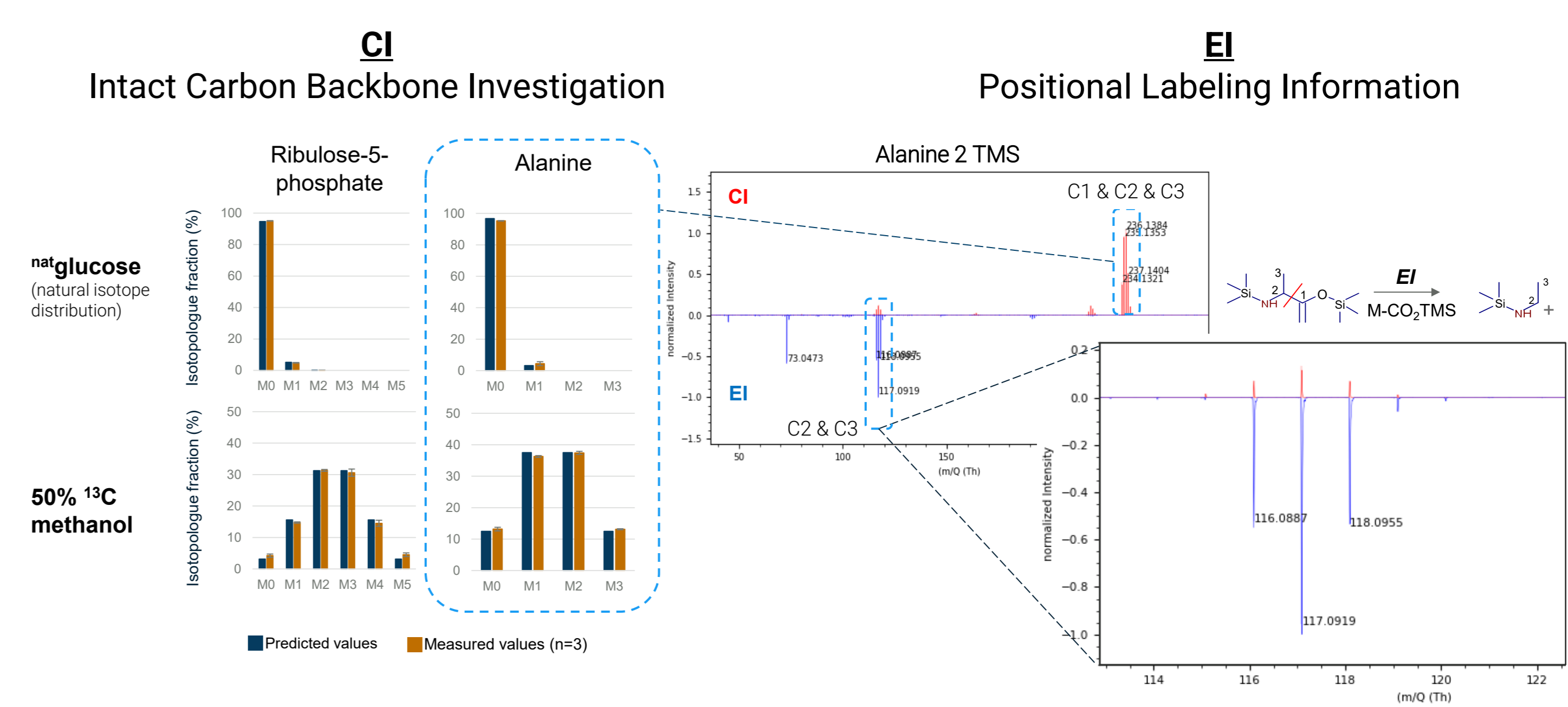
### EI vs. CI – Sensitivity for Hexose Phosphates

Classic CI sources often suffer from very low sensitivities in comparison to EI and can even lack the generation of molecular information. The novel HRP CI source shows a strongly improved performance compared to other CI sources and depending on the compound class can even outperform common EI sensitivities.



Accurate mass Extracted Ion Chromatograms (EIC) of glucose- and mannose-6-phosphates (G6P (~0.8 μM) and M6P (~0.2 μM)) derivatives in yeast extract. CI EIC (red, head) displays the  $M+H^+$  ( $m/z$  736.353). The EI EIC (blue, dashed, tail) displays the common quantifier ion of G6P and M6P ( $m/z$  471.169). For better signal intensity comparison, the EI EIC had to be multiplied by a factor of 10 to match the same range of the CI signal intensity.

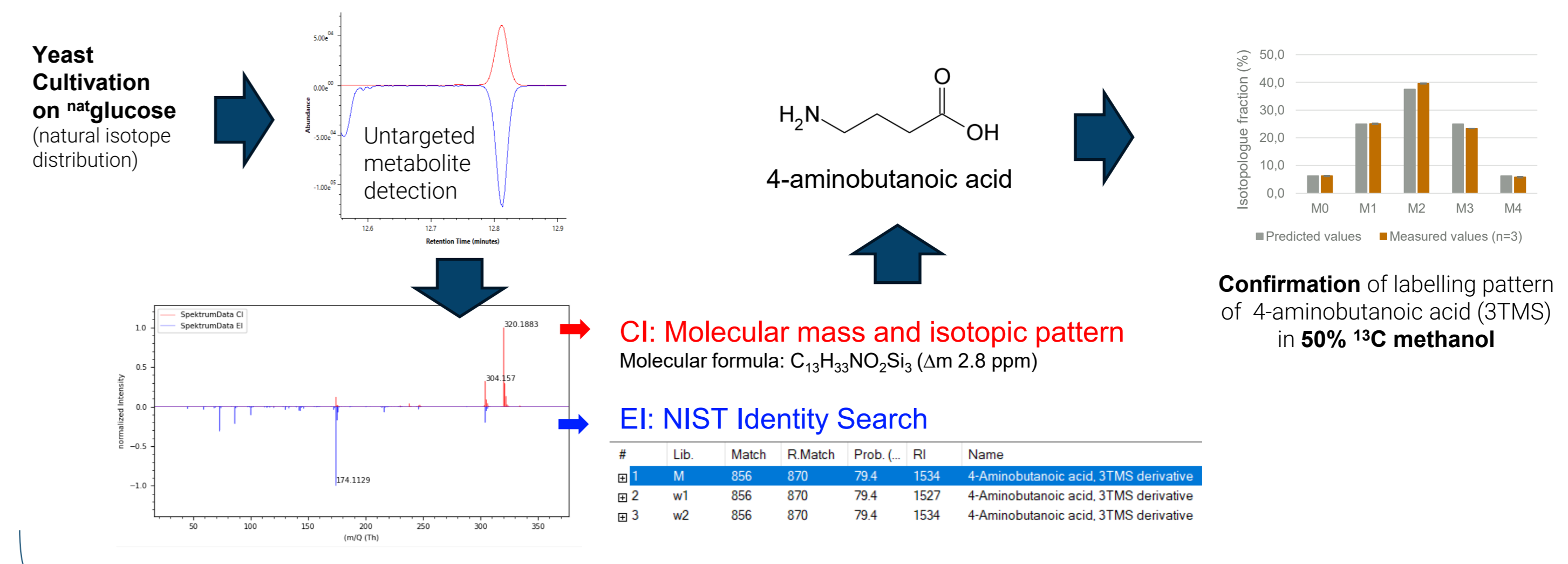
### Improved Isotopologue Analysis due to simultaneous CI & EI Data



Calculated (blue) and CI measured (brown) isotopologue fractions of the intact carbon backbone of alanine and ribulose-5-phosphate TMS derivatives. The different EI fragments give information on the extent of labeling for selected C atoms of the backbone.

Combined sensitive EI & CI information provides accurate insights on active metabolic pathways.

### Retrospective Untargeted Analysis – Metabolite ID beyond the target list



Extension of the targeted metabolite list via untargeted detection and identification of unknowns at high level of confidence by combining EI & CI data.

## CONCLUSIONS

- The ecTOF offers a **unique combination of established and novel technology**
- Simultaneous acquisition of EI and CI spectra in a single GC run** enables reduced analysis time while efficiently generating perfectly aligned structural & molecular information
- Combination of structural data (EI) and molecular information (CI) allows **untargeted analysis with high levels of confidence in compound ID**
- Trueness evaluation with yeast labeled with 50% <sup>13</sup>C methanol confirms **accurate measurement of isotopologue ratios** = prerequisite for metabolic flux analysis

ecTOF

Acknowledgement:



Innovation with Integrity