Supplementary Appendix 1 for

A Pareto approach to resolve the conflict between
information gain and experimental costs:
Multiple-criteria design of carbon labeling experiments

Measurement Models

Katharina Nöh, Sebastian Niedenführ, Martin Beyß, Wolfgang Wiechert
k.noeh@fz-juelich.de

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1. Analytical platforms – overview

This section briefly introduces relevant terminology used throughout, while the interested reader is referred to extensive expert reviews and textbooks for more details [1,2].

NMR is a noninvasive technology that distinguishes isotopes by their magnetic properties. The separation relies on frequency shifts induced by protons (¹H-NMR), carbon nuclei (¹³C-NMR) separately or in combination (heteronuclear NMR). ¹H-NMR measures the positional fractional enrichment in a single carbon position and therewith provides specific relative label information [3]. ¹³C-NMR quantifies local patterns of neighboring labeled carbon atoms [4,5]. NMR based technologies deliver measurement information with high chemical specificity, but devices are high-priced and suffer from low sensitivity implying the need for large sample sizes and comparably long acquisition times.

Complementary to NMR, (tandem) MS instruments measure sums of isotopomers or isotopomer fragments sharing identical masses (“cumulative enrichments”) [6]. In particular, in the collision-induced fragmentation step within tandem MS procedures, molecules (precursor ions) are cleaved into smaller parts (product ions) which can deliver positional labeling information [7–9]. Typically, MS/(MS) is coupled with a preceding chromatographic separation step, such as gas and liquid chromatography (GC/LC) to increase measurement selectivity. In the field of ¹³C MFA, GC-MS is the predominant platform applied. GC-combustion-isotope ratio mass spectrometry (GC-C-IRMS) is a highly specialized technique to quantify the total fraction of labeled and unlabeled carbon content per molecule [10–12], with detection levels two orders of magnitude lower than GC-MS while requiring only very small sample sizes [13]. Besides these hyphenated techniques, also “direct” matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS and Fourier transform-ion cyclotron resonance mass spectrometry (FT-ICR-MS) have been applied in ¹³C MFA, although use cases are rare [14,15].

NMR and MS based analytical platforms differ in their ability to resolve certain chemical classes of metabolites, detectable analyte concentrations and achievable fragmentation patterns (S1 Table A).

Table A. Analytical platforms typically used in ¹³C MFA studies along with the reported target analyte classes.

| Analyte spectrum | Organic acids | Sugars | Amino acids | Comment |
|------------------|---------------|--------|-------------|---------|
| ¹H-NMR           | -             | -      | [3]         | Fine structures; mostly from hydrolyzed cellular proteins due to limited sensitivity (> 1-2 nmol); non-destructive technology; costly instruments; long acquisition times and complex analysis |
| ¹³C-NMR          | -             | -      | [4,16–20]   | Fine structures; mostly from hydrolyzed cellular proteins due to low sensitivity; nucleosides; non-destructive technology; costly instruments; long acquisition times and complex analysis |
| Technique       | Refs.                | Details                                                                                                                                 |
|-----------------|----------------------|------------------------------------------------------------------------------------------------------------------------------------------|
| GC-MS           | [21,22] [23] [17,23–31] | Mass isotopomers; derivatization and bias correction step for natural abundant isotopes mandatory; indirect fragment labeling information; very robust and reproducible; easy analysis; most abundant technique for $^{13}$C MFA; good analytical cost-benefit ratio |
| GC-C-IRMS       | - [-] [10,32]        | Fractional enrichment; especially suited for low labeling content; extensive isolation; small metabolite spectrum                              |
| LC-MS           | [33–35] [34,35] [7,34,36] | Mass isotopomers; non-trivial data analysis; measures against ion suppression required; high sensitivity and selectivity; direct labeling information |
| LC-MS/MS        | [7,37] [34,35,37] [7,37] | Tandem mass isotopomers; non-trivial and time-consuming data analysis; measures against ion suppression required; highest sensitivity and selectivity; direct fragment labeling information |
| MALDI-TOF-MS    | - [14] [14]         | Crude extracts; direct introduction method; more tolerant to higher salt content samples than electrospray ionization methods; new MALDI matrices minimize the issue of matrix inference; fast analysis |
| FT-ICR-MS       | - [38] [15,39]      | Ultra-high resolution and mass accuracies better than 0.2 ppm; fast analysis < 5 min per sample                                             |

With respect to their use in the context of $^{13}$C MFA, comparative investigations on the inter-platform information content of CLEs for $^{13}$C MFA are scarce. Jeffrey et al. compared $^{13}$C-NMR, GC-MS, and GC-MS/MS for measuring the $^{13}$C-fractional enrichment of glutamate resulting in the statement that flux results benefit from the increased number of MS/MS measurement as compared to those obtained by $^{13}$C-NMR and GC-MS [40]. Single and tandem LC- and GC-MS, respectively, have been applied in a network-wide manner in [7,41] and it was shown that tandem MS indeed gives a better overall flux determinacy compared to single MS. On the other hand, different analytical techniques have been combined to increase the coverage of the metabolite and isotopomer spectrum. For instance, GC-MS, $^1$H- and $^{13}$C-NMR by McKinlay et al. for the capnophilic bacterium *Actinobacillus succinogenes* [42], GC-MS, LC-MS and $^{13}$C-NMR for $^{13}$C MFA in *Saccharomyces cerevisiae* [35], and LC-MS with $^{13}$C-NMR in *Penicillium chrysogenum* [43]. Recently, GC- and LC-MS derived labeling data were jointly used to resolve metabolic fluxes in *Pseudomonas fluorescens* [44].
2. Compilation of calibrated device-specific measurement error models

To arrive at realistic error approximations for the measurement covariance matrix, data from studies featuring different organisms, platforms and various labeling contents are collected [3,10,19,20,23,25,27,34,36,37,42,45–49]. For six analytical platforms, namely GC-MS, LC-MS, LC-MS/MS, $^{13}$C-NMR, $^1$H-NMR, and GC-C-IRMS, published measurements and their corresponding standard deviations were extracted (cf. Sec. 3-8). The measurements are assumed to be corrected for natural abundance. Each measurement group was considered only once, also if it is acquired by different analytical methods. In order to prevent over-optimistic predictions, we used conservative, i.e., on average higher error estimates than reported in, e.g., [15,45]. For the same reason, very specialized setups are not considered in the survey (e.g.[38]).

Not unexpectedly, the reported data showed large variances. Similar to the approach in Dauner et al. for $^{13}$C-NMR [18], we proposed a linear regression between the measurements’ standard deviation and the observed signal:

$$\sigma_{meas}^{dev,li} = b_1^{dev} \cdot \eta + b_2^{dev}$$

where the device-specific regression coefficients $b_1^{dev}$, $b_2^{dev}$ are calibrated with all data collected for the device, i.e., across all measurement groups. For $^1$H-NMR and GC-C-IRMS only few labeling data sets are publicly available, all with only low label incorporation levels questioning the validity of the error models for these two platforms for higher levels of fractional enrichments.

Results of the linear regressions are shown in S1 Fig A, including the values for the coefficient pairs. In summary, the regression lines of the different platforms show only minor differences in their slopes. Roughly, in regions with low label enrichment GC-C-IRMS and LC-MS/MS errors are modeled to be most accurate while for $^{13}$C-NMR the error remains nearly constant over the whole possible labeling range. With these measurement error models at hand, errors become predictable in dependence of the analytes’ labeling states. As a consequence, the variances in the main diagonal of the measurement covariance matrix depend on the labeling fraction and are, thus, heteroscedastic.
Fig A. Comparison of device-specific error models for fractional labeling measurements. Underlying data are collected from studies utilizing different organisms and labeling contents (see S1 Sec. 3-8 for the specifications). Standard deviations are determined by linear regression, i.e., by fitting a linear error model $\sigma = a \cdot \eta + b$ with slope ($a$) and y-axis intercept ($b$). Here, $b$ represents the baseline error. For $^1$H-NMR only data sets with a low abundance of $^{13}$C labeling content are available. Therefore, the error model is considered realistic only for labeling fractions of less than 30% $^{13}$C incorporation, while errors of measurements with higher labeling content are linearly extrapolated, as indicated by the change in line thickness.
3. Gas Chromatography-Mass Spectrometry (GC-MS)

3.1 Measurement specification

Table B. GC-MS measurement group specification.

| Metabolite          | # Carbons | Measurement specification | Fragment and/or mass | References (exemplary) |
|---------------------|-----------|---------------------------|----------------------|------------------------|
| Alanine ALA         | 3         | ALA#M0,1,2,3              | M-57, 260            | [23], [45], [27], [25], [46], [42], [47] |
|                     |           | ALA[2-3]#M0,1,2           | M-85, 232            | [23], [45], [27], [25], [46], [42], [47] |
|                     |           |                           | M-159                | [25]                   |
| Arginine ARG*       | 6         | ARG#M0,1,2,3,4,5          | 442                  | [23]                   |
| Aspartate ASP       | 4         | ASP#M0,1,2,3,4            | M-57, 418            | [23], [45], [27], [46], [47] |
|                     |           |                           | M-15, 460            | [27]                   |
|                     |           | ASP[2-4]#M0,1,2,3         | M-85, 390            | [23], [45], [46], [47] |
|                     |           |                           | M-159, 316           | [23], [27]             |
|                     |           | ASP[1-2]#M0,1,2           | f302, 302            | [45], [46], [47]       |
| Aspartate/Asparagine ASX* | 4       | ASX#M0,1,2,3,4            | M-57, 418            | [25], [42]             |
|                     |           | ASX[2-4]#M0,1,2,3         | M-85, 390            | [25], [42]             |
|                     |           |                           | M-159, 316           | [25], [42]             |
|                     |           | ASX[1-2]#M0,1,2           | f302, 302            | [25], [42]             |
| Fumarate FUM*       | 4         | FUM#M0,1,2,3,4            | M-57                 | [42]                   |
| Glycine GLY         | 2         | GLY#M0,1,2                | M-57, 246            | [23], [45], [27], [25], [46], [42], [47] |
|                     |           | GLY[2]#M0,1*              | M-85, 218            | [23], [45], [27], [25], [46], [42], [47] |
| Glutamate GLU       | 5         | GLU#M0,1,2,3,4,5          | M-57, 432            | [23], [45], [27], [46], [47] |
|                     |           | GLU[2-5]#M0,1,2,3,4       | M-85, 404            | [45], [27], [46], [47] |
|                     |           |                           | M-159, 330           | [23], [27], [46]       |
| Glutamate/Glutamine GLX* | 5   | GLX#M0,1,2,3,4,5          | M-57                 | [25], [42]             |
|                     |           | GLX[2-5]#M0,1,2,3,4       | M-85                 | [25], [42]             |
|                     |           |                           | M-159                | [25], [42]             |
|                     |           | GLX[1-2]#M0,1,2           | f302, 302            | [25]                   |
| Amino Acid | Short Form | N Terminals | M-Terminals | References |
|------------|------------|-------------|-------------|------------|
| Glutamine  | GLN*       | 5           | GLN#M0,1,2,3,4,5 | M-57, 431 [46] |
| Histidine  | HIS*       | 6           | HIS[2-6]#M0,1,2,3,4,5 | n.a. [45] |
| Isoleucine | ILE        | 6           | ILE#M0,1,2,3,4,5,6 | n.a. [45], [47] |
|            |            |             | ILE[2-6]#M0,1,2,3,4,5 | M-85, 274 [45], [27], [25], [46], [42], [47] |
|            |            |             |             | M-159, 200 [27], [25], [46], [42] |
| Leucine    | LEU        | 6           | LEU#M0,1,2,3,4,5,6 | [47] |
|            |            |             | LEU[2-6]#M0,1,2,3,4,5 | M-85, 274 [45], [27], [25], [46], [42], [47] |
|            |            |             |             | M-159, 200 [27], [25], [42] |
| Lysine     | LYS        | 6           | LYS#M0,1,2,3,4,5,6 | M-57 [25], [47] |
|            |            |             | LYS[2-6]#M0,1,2,3,4,5 | M-159, 329 [27], [25], [47] |
|            |            |             | LYS[1-2]#M0,1 | f302, 302 [25] |
| Methionine | MET        | 5           | MET#M0,1,2,3,4,5 | M-57, 320 [27], [25], [46], [42] |
|            |            |             | MET[2-5]#M0,1,2,3,4 | M-85, 292 [27], [25], [46], [42] |
|            |            |             |             | M-159, 218 [27], [25], [46] |
| Phenylalanine | PHE | 9       | PHE#M0,1,2,3,4,5,6,7,8,9 | M-57, 336 [23], [45], [27], [46], [42], [47] |
|            |            |             | PHE[2-9] #M0,1,2,3,4,5,6,7,8 | M-85, 308 [45], [27], [25], [46], [42], [47] |
|            |            |             |             | M-159, 234 [23], [27], [25], [46] |
|            |            |             | PHE[3-9] #M0,1,2,3,4,5,6,7 | sc [25] |
|            |            |             | PHE[1-2]#M0,1,2 | f302, 302 [23], [45], [25], [46], [42], [47] |
| Proline    | PRO        | 5           | PRO#M0,1,2,3,4,5 | M-57 [45], [25], [47] |
|            |            |             | PRO[2-5]#M0,1,2,3,4 | M-85 [45], [25], [47] |
|            |            |             |             | M-159, 184 [27], [25], [42] |
| Serine     | SER        | 3           | SER#M0,1,2,3 | M-57, 390 [23], [45], [27], [25], [46], [42], [47] |
|            |            |             | SER[2-3]#M0,1,2 | M-85, 362 [23], [45], [27], [46], [42], [47] |
|            |            |             |             | M-159, 27 [27], [25], [46], [42], [47] |
| Metabolite | MS Measurement | Carbons | MS Measurement | Ref.   |
|------------|----------------|---------|----------------|--------|
| Succinate  | SUC*           | 4       | SUC#M0,1,2,3,4 | M-15   |
|            |                |         |                | [42]   |
| Threonine  | THR            | 4       | THR#M0,1,2,3,4 | M-57,  |
|            |                |         |                | 404,   |
|            |                |         |                | [23],  |
|            |                |         |                | [45],  |
|            |                |         |                | [47],  |
|            |                |         |                | [42],  |
| Tyrosine   | TYR            | 9       | TYR#M0,1,2,3,4,5,6,7,8,9* | M-159 |
|            |                |         |                | [25]   |
|            |                |         |                | [45]   |
|            |                |         |                | [47]   |
|            | TYR[2-9]#M0,1,2,3,4,5,6,7,8 | M-85   | [23], [45], [47] |
|            |                |         |                | [25]   |
|            | TYR[1-2]#M0,1,2 | f302,302 | [23], [45], [46], [47] |
|            |                |         |                | [42],  |
|            |                |         |                | [47]   |
|            | VAL#M0,1,2,3,4,5 | M-57   | [23], [45], [47] |
|            |                |         |                | [27],  |
|            |                |         |                | [25]   |
|            |                |         |                | [46],  |
|            |                |         |                | [42],  |
|            | VAL[2-5]#M0,1,2,3,4 | M-85   | [23], [45], [47] |
|            |                |         |                | [27],  |
|            |                |         |                | [25]   |
|            |                |         |                | [46],  |
|            |                |         |                | [47]   |
|            |                |         |                | [23],  |
|            |                |         |                | [27]   |
|            |                |         |                | [42]   |
|            | VAL[1-2]#M0,1,2* | f302,302 | [45], [47] |

*: metabolite not present in the reaction network or not used in the study
n.a.: information not available

The MS measurement specification METAB#M0,1,2,3 describes a MS measurement of the full molecule of the metabolite pool METAB having three carbon atoms. If only a certain molecule fragment is observed, the carbon-range is specified that contains the labeling positions, e.g., METAB[2-3]#M0,1,2.
Fig B. Network and GC-MS labeling measurements. Each measurement group is represented by a circle giving the number of measured fractions. All metabolic network diagrams were drawn with the software OMIX [50]. Visualization of data was done with tailored OVL scripts.
3.2 Measurement model

Mass isotopomers can be expressed – up to a normalization factor – as a linear combination of isotopomer fractions [51], as shown here for the C3 metabolite pyruvate as an example:

\[
\begin{pmatrix}
PYR # M0 \\
PYR # M1 \\
PYR # M2 \\
PYR # M3
\end{pmatrix}
= 
\begin{pmatrix}
1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 1 & 1 & 0 & 1 & 0 & 0 & 0 \\
0 & 0 & 0 & 1 & 0 & 1 & 1 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & 1
\end{pmatrix}
\cdot
\begin{pmatrix}
\mathbf{x}_{\infty} \\
\mathbf{x}_{\infty} \\
\mathbf{x}_{\infty} \\
\mathbf{x}_{\infty} \\
\mathbf{x}_{\infty} \\
\mathbf{x}_{\infty} \\
\mathbf{x}_{\infty} \\
\mathbf{x}_{\infty}
\end{pmatrix}
= 
\mathbf{M}_{PYR,MS} \cdot \mathbf{x}_{PYR}
\]

where \( \mathbf{x}_{PYR} \) denotes the vector of isotopomer fractions of the metabolite PYR.

3.3 Measurement error model

Fig C. Error model for GC-MS based labeling measurements compiled from published data sets (S1 Table B). Standard deviations were determined by linear regression, i.e., by fitting the linear error model \( \sigma = b + m \cdot \eta \) with slope (m) and y-axis intercept (b) to the data resulting in \( \sigma = 0.006653 + 0.04119 \cdot \eta \).
4. Liquid Chromatography-Mass Spectrometry (LC-MS)

4.1 Measurement specification

Table C: LC-MS measurement group specification.

| Metabolite                                   | # Carbons | Measurement specification | References (exemplary) |
|----------------------------------------------|-----------|---------------------------|------------------------|
| 2-P-Glycerate + 3-P-Glycerate                | 23PG*     | 23PG#M0,1,2,3             | [47], [49], [34]      |
| 6-Phosphogluconate                          | 6PG*      | 6PG#M0,1,2,3,4,5,6        | [47], [49], [34]      |
| 2-oxoglutarate                              | AKG       | AKG# M0,1,2,3,4,5         | [47]                  |
| Alanine                                     | ALA       | ALA#M0,1,2,3              | [46], [36]            |
| Arginine                                    | ARG       | ARG#M0,1,2,3,4,5,6        | [52]                  |
| Asparagine                                  | ASN       | ASN#M0,1,2,3,4            | [46], [36]            |
| Aspartate                                   | ASP       | ASP#M0,1,2,3,4            | [46]                  |
| Cysteine                                    | CYS       | CYS#M0,1,2,3              | [37]                  |
| Dihydroxyacetone-phosphate                  | DHAP      | DHAP#M0,1,2,3             | [37]                  |
| Erythrose-4-phosphate                       | E4P       | E4P#M0,1,2,3,4            | [34]                  |
| Fructose-6-phosphate                        | F6P       | F6P#M0,1,2,3,4,5,6        | [47], [34]            |
| Fructose-1,6-phosphate                      | FBP       | FBP#M0,1,2,3,4,5,6        | [47], [49], [34]      |
| Fumarate                                    | FUM       | FUM# M0,1,2,3,4           | [47]                  |
| Glucose-1-phosphate                         | G1P*      | G1P#M0,1,2,3,4,5,6        | [34]                  |
| Glucose-6-phosphate                         | G6P       | G6P#M0,1,2,3,4,5,6        | [47], [34]            |
| Glutamine                                   | GLN       | GLN#M0,1,2,3,4,5          | [36]                  |
| Glutamate                                   | GLU       | GLU#M0,1,2,3,4,5          | [46], [36]            |
| Glyceraldehyde-3-phosphate                  | GAP       | GAP#M0,1,2,3              | [37]                  |
| Glycine                                     | GLY       | GLY#M0,1,2                | [46], [36]            |
| Histidine                                   | HIS       | HIS#M0,1,2,3,4,5,6        | [37]                  |
| Isoleucine                                  | ILE       | ILE#M0,1,2,3,4,5,6        | [37]                  |
| Leucine                                     | LEU       | LEU#M0,1,2,3,4,5,6        | [37]                  |
| Lysine                                      | LYS       | LYS#M0,1,2,3,4,5,6        | [37]                  |
| Malate                                      | MAL       | MAL# M0,1,2,3,4           | [47]                  |
| Methionine                                  | MET       | MET#M0,1,2,3,4,5          | [46]                  |
| Oxaloacetate                                | OAA       | OAA#M0,1,2,3,4,5          | [37]                  |
| Ribose-5-phosphate + Ribulose-5-phosphate + | RU5P      | RU5P#M0,1,2,3,4,5         | [47], [49], [34]      |
| Xylulose-5-phosphate                        |           |                           |                       |
| Phosphoenolpyruvate                         | PEP       | PEP# M0,1,2,3             | [47]                  |
| Phenylalanine                               | PHE       | PHE# M0,1,2,3,4,5,6,7,8,9| [46], [36]            |
| Proline                                     | PRO       | PRO#M0,1,2,3,4,5          | [37]                  |
| Pyruvate                                    | PYR       | PYR# M0,1,2,3             | [37]                  |
| Metabolite                | Code | Value | MS Measurement Specification |
|--------------------------|------|-------|-------------------------------|
| Sedoheptulose-7-phosphate| S7P  | 7     | S7P#M0,1,2,3,4,5,6,7           |
| Serine                   | SER  | 3     | SER#M0,1,2,3                   |
| Succinate                | SUC  | 4     | SUC# M0,1,2,3,4                |
| Threonine                | THR  | 4     | THR# M0,1,2,3,4                |
| Tryptophane              | TRP* | 11    | TRP# M0,1,2,3,4,5,6,7,8,9,10,11|
| Tyrosine                 | TYR  | 9     | TYR# M0,1,2,3,4,5,6,7,8,9      |
| Valine                   | VAL  | 5     | VAL# M0,1,2,3,4,5              |

*: metabolite not present in the reaction network or not used in the study

The MS measurement specification METAB#M0,1,2,3 describes a MS measurement of the full molecule of the metabolite pool METAB having three carbon atoms.
Fig D. Network and LC-MS labeling measurements. Each measurement group is represented by a circle giving the number of measured fractions.
4.2 Measurement model
same as in S1 Sec 3.2.

4.3 Measurement error model

Fig E. Error model for LC-MS based labeling measurements compiled from published data sets (S1 Table C). Standard deviations were determined by linear regression, i.e., by fitting the linear error model \( \sigma = b + m \cdot \eta \) with slope \( m \) and y-axis intercept \( b \) to the data resulting in \( \sigma = 0.008839 + 0.018934 \cdot \eta \).
5. Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

5.1 Measurement specification

Table D. LC-MS/MS measurement group specification.

| Metabolite                        | # Carbons | Measurement specification            | Reference (exemplary) |
|-----------------------------------|-----------|--------------------------------------|-----------------------|
| 2-phosphate-glycerate + 3-phosphate-glycerate | 23PG*     | 23PG[1-3:1-3]#M(0,0)(1,1)(2,2)(3,3)   | [37]                  |
| 6-phosphogluconate                | 6PG*      | 6PG[1:6:1:6]#M(0,0)(1,1)(2,2)(3,3)(4,4)(5,5)(6,6) | [37]                  |
| 2-oxoglutarate                    | AKG       | AKG[1-5:1-4]#M(0,0)(1,1)(2,1)(2,2)(3,3)(4,3) (4,4) (5,4) | [37]                  |
| Alanine                           | ALA       | ALA[1-3:2-3]#M(0,0)(1,1)(2,1)(2,2)(3,2) | [37]                  |
| Arginine                          | ARG       | ARG[1-6:1-5]#M(0,0)(1,0)(1,1)(2,1)(2,2)(3,3)(4,3) (4,4) (5,4) (5,5) (6,5) | [37]                  |
| Asparagine                        | ASN       | ASN[1-4:2-4]#M(0,0)(1,0)(1,1)(2,1)(2,2)(3,3)(4,3) | [37]                  |
| Aspartate                         | ASP       | ASP[1-4:1-2]#M(0,0)(1,0)(1,1)(2,0)(2,1)(2,2)(3,1)(3,2) (4,2) | [37]                  |
| Citrate + Isocitrate              | CIT*      | CIT[1-6:2-6]#M(0,0)(1,0)(1,1)(2,1)(2,2)(3,2)(3,3)(4,3) (4,4) (5,4) (5,5) (6,5) | [37]                  |
| Cysteine                          | CYS       | CYS[1-3:2-3]#M(0,0)(1,0)(1,1)(2,1)(2,2)(3,2) | [37]                  |
| Dihydroxyacetone-phosphosphate    | DHAP      | DHAP[1-3:1-3]#M(0,0)(1,1)(2,2)(3,3) | [37]                  |
| Erythrose-4-phosphate             | E4P       | E4P[1-4:1-4]#M(0,0)(1,1)(2,2)(3,3)(4,4) | [37]                  |
| Fumarate                          | FUM       | FUM[1-4:1-3]#M(0,0)(1,0)(1,1)(2,1)(2,2) (3,2)(3,3)(4,3) (1-3)=2-4 | [37]                  |
| Fructose-6-phosphate              | F6P       | F6P[1-6:1-6]#M(0,0)(1,1)(2,2)(3,3)(4,4)(5,5)(6,6) | [37]                  |
| Fructose-1,6-phosphate            | FBP       | FBP[1-6:1-6]#M(0,0)(1,1)(2,2)(3,3)(4,4)(5,5)(6,6) | [37]                  |
| Glucose-6-phosphate               | G6P       | G6P[1-6:1-6]#M(0,0)(1,1)(2,2)(3,3)(4,4)(5,5)(6,6) | [37]                  |
| Glycer-aldehyde-3-phosphate       | GAP       | GAP[1-3:1-3]#M(0,0)(1,1)(2,2)(3,3) | [37]                  |
| Amino Acid       | Abbreviation | Count | Formula | Spectrum |
|------------------|--------------|-------|---------|----------|
| Glutamine        | GLN          | 5     | GLN[1-5:2-5]#M(0,0)(1,0)(1,1)(2,1)(2,2)(3,2)(3,3)(4,3)(4,4)(5,4) | [37] |
| Glutamate        | GLU          | 5     | GLU[1-5:2-5]#M(0,0)(1,0)(1,1)(2,1)(2,2)(3,2)(3,3)(4,3)(4,4)(5,4) | [37] |
| Glycine          | GLY          | 2     | GLY[1-2:1]#M(0,0)(1,0)(1,1)(2,1) | [37] |
| Histidine        | HIS          | 6     | HIS[1-6:2-6]#M(0,0)(1,0)(1,1)(2,1)(2,2)(3,2)(3,3)(4,3)(4,4)(5,4)(5,5)(6,5) | [37] |
| Isoleucine       | LEU          | 6     | LEU[1-6:2-6]#M(0,0)(1,0)(1,1)(2,1)(2,2)(3,2)(3,3)(4,3)(4,4)(5,4)(5,5)(6,5) | [37] |
| Leucine          | ILEU         | 6     | ILEU[1-6:2-6]#M(0,0)(1,0)(1,1)(2,1)(2,2)(3,2)(3,3)(4,3)(4,4)(5,4)(5,5)(6,5) | [37] |
| Lysine           | LYS          | 6     | LYS[1-6:1-6]#M(0,0)(1,1)(2,2)(3,3)(4,4)(5,5)(6,6) | [37] |
| Malate           | MAL          | 4     | MAL[1-4:1-4]#M(0,0)(1,1)(2,2)(3,3)(4,4) | [37] |
| Methionine       | MET          | 5     | MET[1-5:2-5]#M(0,0)(1,0)(1,1)(2,1)(2,2)(3,2)(3,3)(4,3)(4,4)(5,4) | [37] |
| Oxaloacetate     | OAA          | 4     | OAA[1-4:1-3]#M(0,0)(1,0)(1,1)(2,1)(2,2)(3,2)(3,3)(4,3) | [37] |
| Phosphoenolpyruvate| PEP       | 3     | PEP[1-3:1-3]#M(0,0)(1,1)(2,2)(3,3) | [37] |
| Phenylalanine    | PHE          | 9     | PHE[1-9:2-9]#M(0,0)(1,0)(1,1)(2,1)(2,2)(3,2)(3,3)(4,3)(4,4)(5,4)(5,5)(5,6)(5,5)(6,6)(6,6)(7,7)(7,7)(7,7)(7,7)(7,8)(8,8)(9,8) | [37] |
| Proline          | PRO          | 5     | PRO[1-5:2-5]#M(0,0)(1,0)(1,1)(2,1)(2,2)(3,2)(3,3)(4,3)(4,4)(5,4) | [37] |
| Pyruvate         | PYR          | 3     | PYR[1-3:2-3]#M(0,0)(1,0)(1,1)(2,1)(2,2)(3,2) | [37] |
| Ribose-5-P       | R5P*         | 5     | R5P[1-5:1-5]#M(0,0)(1,1)(2,2)(3,3)(4,4)(5,5) | [37] |
| Ribulose-5-P + Xylulose-5-P | RU5P | 5     | RU5P[1-5:1-5]#M(0,0)(1,1)(2,2)(3,3)(4,4)(5,5) | [37] |
| Sedoheptulose-7-phosphate | S7P | 7     | S7P[1-7:1-7]#M(0,0)(1,1)(2,2)(3,3)(4,4)(5,5)(6,6)(7,7) | [37] |
| Serine           | SER          | 3     | SER[1-3:2-3]#M(0,0)(1,0)(1,1)(2,1)(2,2)(3,2) | [37] |
| Succinate        | SUC          | 4     | SUC[1-4:1-3]#M(0,0)(1,0)(1,1)(2,1)(2,2)(3,2)(3,3)(4,3)(1-3)=[2-4] | [37] |
| Homoserine/Threonine | THR    | 4     | THR[1-4:2-4]#M(0,0)(1,0)(1,1)(2,1)(2,2)(3,2)(3,3)(4,3) | [37] |
| Metabolite     | Abbreviation | Charge | Mass Spectrum | Mass Increments | Reference |
|---------------|--------------|--------|---------------|-----------------|-----------|
| Tryptophane   | TRP*         | 11     | TRP[1-11:1-11]| #M(0,0)(1,1)(2,2)(3,3)(4,4)(5,5)(6,6)(7,7)(8,8)(9,9)(10,10)(11,11) | [37]      |
| Tyrosine      | TYR          | 9      | TYR[1-9:2-9]  | #M(0,0)(1,0)(1,1)(2,1)(2,2)(3,2)(3,3)(4,3)(4,4)(5,4)(5,5)(6,5)(6,6)(7,6)(7,7)(8,7)(8,8)(9,8) | [37]      |
| Valine        | VAL          | 5      | VAL[1-5:2-5]  | #M(0,0)(1,0)(1,1)(2,1)(2,2)(3,2)(3,3)(4,3)(4,4)(5,4) | [37]      |

*: metabolite not present in the reaction network or not used in the study

The tandem-MS measurement group specification METAB[1-3:2,3]#M0,1,2,3 of a C3 metabolite METAB describes a tandem-MS measurement on the full (mother) molecule METAB and on its (daughter) fragment consisting of the second and third atom position. The list of pairs of mass traces following #M denotes the mass-increments of the mother and daughter fragments.
Fig F. Network and LC-MS/MS labeling measurements. Each measurement group is represented by a circle giving the number of measured fractions.
5.2 Measurement model

Tandem mass isotopomers can be expressed – up to a normalization factor – as a linear combination of isotopomer fractions [7], as shown here for the C3 metabolite pyruvate as an example:

\[
\begin{align*}
&PYR[1 - 3 : 2 - 3] \# M(0,0) = \begin{pmatrix} 1 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 1 & 0 & 0 & 0 \\ 0 & 1 & 1 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 1 & 1 & 0 \\ 0 & 0 & 1 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 1 \end{pmatrix} \cdot \begin{pmatrix} x_{\infty} \\ x_{\infty} \\ x_{\infty} \\ x_{\infty} \\ x_{\infty} \\ x_{\infty} \end{pmatrix} = M_{PYR,MSMS} \cdot x_{PYR}
\end{align*}
\]

where \( x_{PYR} \) denotes the vector of isotopomer fractions of the metabolite PYR. In this case C1-3 denotes the mother ion and C2-3 the daughter ion.

5.3 Measurement error model

Fig G. Error model for LC-MS/MS based labeling measurements compiled from published data sets (S1 Table D). Standard deviations were determined by linear regression, i.e., by fitting the linear error model \( \sigma = b + m \cdot \eta \) with slope \( m \) and y-axis intercept \( b \) to the data resulting in \( \sigma = 0.001696+0.016496 \cdot \eta \).
6. $^{13}$C-Nuclear Magnetic Resonance Spectrometry ($^{13}$C-NMR)

6.1 Measurement specification

Table E. 2D-$^{13}$C NMR measurement group specification.

| Metabolite | Analytical technique | Measurement specification | References |
|------------|----------------------|---------------------------|------------|
| Alanine    | ALA                  | $^{1}$H-$^{13}$C HSQC ALA#S2,DL2,DR2,DD2 ALA#S3,DL3 | [20], [48] |
|            |                      | $^{1}$H-$^{13}$C COSY ALA#S2,DL2,DR2,DD2 ALA#S3,DL3 | [47], [19] |
| Arginine   | ARG                  | $^{1}$H-$^{13}$C HSQC ARG#S3,DL3,T3 ARG#S5,DL5 | [48] |
|            |                      | $^{1}$H-$^{13}$C COSY ARG#S3,DL3,DD3 ARG#S4,DL4,DD4 ARG#S5,DL5 | [19] |
|            |                      | ARG#S3,DL3,T3 ARG#S5,DL5 | [47] |
| Aspartate  | ASP                  | $^{1}$H-$^{13}$C HSQC ASP#S2,DL2,DR2,DD2 ASP#S3,DL3,DR3,DD3 | [20], [48] |
|            |                      | $^{1}$H-$^{13}$C COSY ASP#S2,DL2,DR2,DD2 ASP#S3,DL3,DR3,DD3 | [47] |
| Aspartate/ Asparagine | ASX* | $^{1}$H-$^{13}$C COSY ASX#S2,DL2,DR2,DD2 ASX#S3,DL3,DR3,DD3 | [19] |
| Cysteine   | CYS                  | $^{1}$H-$^{13}$C COSY CYS#S2,DL2,DR2,DD2 CYS#S3,DL3 | [47] |
| Glutamate  | GLU                  | $^{1}$H-$^{13}$C HSQC GLU#S2,DL2,DR2,DD2 GLU#S3,DL3,DD3 GLU#S4,DL4,DR4,DD4 | [20] |
|            |                      | $^{1}$H-$^{13}$C COSY GLU#S2,DL2,DR2,DD2 GLU#S3,DL3,T3 GLU#S4,DL4,DR4,DD4 | [47] |
| Glutamate/ Glutamine | GLX* | $^{1}$H-$^{13}$C COSY GLX#S2,DL2,DR2,DD2 GLX#S3,DL3,DD3 GLX#S4,DL4,DR4,DD4 | [19] |
| Glycine    | GLY                  | $^{1}$H-$^{13}$C HSQC GLY#S2,DL2 | [20], [48] |
|            |                      | $^{1}$H-$^{13}$C COSY GLY#S2,DL2 | [47], [19] |
| Histidine  | HIS                  | $^{1}$H-$^{13}$C HSQC HIS#S2,DL2,DR2,DD2 HIS#S3,DL3,DR3,DD3 HIS#S5,DL5 | [20] |
|            |                      | HIS#S3,DL3,DR3,DD3 HIS#S5,DL5 | [48] |
|            |                      | $^{1}$H-$^{13}$C COSY HIS#S2,DL2,DR2,DD2 HIS#S3,DL3,DR3,DD3 | [47], [19] |
|            |                      | HIS#S5,DL5 | |
| Isoleucine | ILE                  | $^{1}$H-$^{13}$C HSQC ILE#S2,DL2,DR2,DD2 ILE#S4,DL4,DD4 ILE#S5,DL5 ILE#S6,DL6 | [48] |
|            |                      | $^{1}$H-$^{13}$C COSY ILE#S2,DL2,DR2,DD2 ILE#S4,DL4,DD4 ILE#S5,DL5 | [19] |
| Protein   | Residue | Experiment          | Assignment               | References |
|-----------|---------|---------------------|--------------------------|------------|
| Leucine   | LEU     | $^1$H-$^13$C HSQC   | LEU#S2,DL2,DR2,DD2       | [20]       |
|           |         |                     | LEU#S3,DL3,DD3           | [47]       |
|           |         |                     | LEU#S5,DL5               | [48]       |
|           |         | $^1$H-$^13$C COSY    | LEU#S2,DL2,DR2,DD2       | [19]       |
|           |         |                     | LEU#S3,DL3,T3            | [47]       |
|           |         |                     | LEU#S5,DL5               | [19]       |
|           |         |                     | LEU#S6,DL6               | [47]       |
| Lysine    | LYS     | $^1$H-$^13$C HSQC   | LYS#S3,DL3,T3            | [48]       |
|           |         |                     | LYS#S4,DL4,T4            | [48]       |
|           |         |                     | LYS#S5,DL5,T5            | [48]       |
|           |         | $^1$H-$^13$C COSY    | LYS#S3,DL3,DD3           | [19]       |
|           |         |                     | LYS#S4,DL4,DD4           | [47]       |
|           |         |                     | LYS#S5,DL5,DD5           | [19]       |
|           |         |                     | LYS#S6,DL6               | [47]       |
| Methionine| MET     | $^1$H-$^13$C COSY    | MET#S2,DL2,DR2,DD2       | [47], [19]|
| Phenylalanine| PHE | $^1$H-$^13$C HSQC   | PHE#S2,DL2,DR2,DD2       | [20]       |
|           |         |                     | PHE#S3,DL3,DD3           | [48]       |
|           |         | $^1$H-$^13$C COSY    | PHE#S2,DL2,DR2,DD2       | [19]       |
|           |         |                     | PHE#S3,DL3,DD3           | [47]       |
| Proline   | PRO     | $^1$H-$^13$C HSQC   | PRO#S2,DL2,DR2,DD2       | [20]       |
|           |         |                     | PRO#S3,DL3,T3            | [48]       |
|           |         | $^1$H-$^13$C COSY    | PRO#S2,DL2,DR2,DD2       | [19]       |
|           |         |                     | PRO#S3,DL3,DD3           | [47]       |
|           |         |                     | PRO#S4,DL4,DD4           | [19]       |
|           |         |                     | PRO#S5,DL5               | [47]       |
| Serine    | SER     | $^1$H-$^13$C HSQC   | SER#S2,DL2,DR2,DD2       | [20], [48]|
|           |         | $^1$H-$^13$C COSY    | SER#S2,DL2,DR2,DD2       | [47], [19]|
|           |         |                     | SER#S3,DL3               | [48]       |
| Threonine | THR     | $^1$H-$^13$C HSQC   | THR#S4,DL4               | [20], [48]|
|           |         | $^1$H-$^13$C COSY    | THR#S2,DL2,DR2,DD2       | [19]       |
|           |         |                     | THR#S3,DL3,DD3           | [47]       |
|           |         |                     | THR#S4,DL4               | [19]       |
|           |         |                     | THR#S2,DL2,DR2,DD2       | [47]       |
|           |         |                     | THR#S3,DL3,T3            | [47]       |
| Tyrosine | THR#S4,DL4 |
|----------|------------|
|          | THR#S5,DL5,T5 [48] |
|          | THR#S5,DL5,DD5 |
|          | THR#S6,DL6,DD6 [20] |
|          | TYR#S2,DL2,DR2,DD2 [47], [19] |
|          | TYR#S3,DL3,DR3,DD3 |

| Valine   | VAL#S2,DL2,DR2,DD2 [20] |
|----------|-------------------------|
|          | VAL#S3,DL3,DD3,DD3 |
|          | VAL#S4,DL4 |
|          | VAL#S5,DL5 [48] |
|          | VAL#S2,DL2,DR2,DD2 [19] |
|          | VAL#S4,DL4 |
|          | VAL#S5,DL5 |
|          | VAL#S2,DL2,DR2,DD2 [47] |
|          | VAL#S4,DL4 |

*: metabolite not represented in the reaction network or used in the study

1H-13C HSQC: two-dimensional heteronuclear single-quantum coherence (13C, 1H)-correlation NMR

1H-13C COSY: two dimensional heteronuclear correlation spectroscopy

NMR fine structures are specified by singlet (S), doublets (D), double doublets (DD), and triplets (T), where DL and DR denote the left and right doublet, respectively, followed by the measured position.
Fig H. Network and $^{13}$C-NMR labeling measurements. Each measurement group is represented by a circle giving the number of measured fractions.
6.2 Measurement model

Multiplet resonances can be expressed – up to a normalization factor – as a linear combination of isotopomer fractions [51], as shown here for the C3 metabolite pyruvate as an example:

\[
\begin{align*}
\begin{pmatrix}
PYR\#S2 \\
PYR\#DL2 \\
PYR\#DR2 \\
PYR\#DD2
\end{pmatrix}
&= 
\begin{pmatrix}
0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 \\
0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & 1
\end{pmatrix}
\cdot
\begin{pmatrix}
x \\
x \\
x \\
x
\end{pmatrix}
\end{align*}
\]

where \(x_{\text{PYR}}\) denotes the vector of isotopomer fractions of the metabolite PYR. PYR[2] indicates that the second carbon atom is observed.

6.3 Measurement error model

Fig I. Error model for \(^{13}\text{C}-\text{NMR}\) based labeling measurements compiled from published data sets (S1 Table E). Standard deviations were determined by linear regression, i.e., by fitting the linear error model \(\sigma = b + m \cdot \eta\) with slope (m) and y-axis intercept (b) to the data resulting in \(\sigma = 0.005959 + 0.000717 \cdot \eta\).
7. $^1$H-Nuclear Magnetic Resonance Spectrometry ($^1$H-NMR)

7.1 Measurement specification

Table F. $^1$H-NMR measurement group specification.

| Metabolite | #carbons | Measurement specification | References |
|------------|----------|---------------------------|------------|
| Acetate    | ACE*     | 2 ACE#P1,2                | [42]       |
| Alanine    | ALA      | 3 ALA#P2,3                | [42], [53] |
| Aspartate  | ASP      | 4 ASP#P2,3                | [53]       |
| Glutamate  | GLU      | 6 GLU#P2,3,4              | [53]       |
| Glycine    | GLY      | 2 GLY#P1,2                | [53]       |
| Isoleucine | ILE      | 6 ILE#P2,5,6              | [53]       |
| Leucine    | LEU      | 6 LEU#P2,3,4,5,6          | [53]       |
| Lysine     | LYS*     | 6 LYS#P2,3,4,5,6          | [53]       |
| Phenylalanine | PHE | 9 PHE#P2,3,4,5,6,7,8,9 | [53]       |
| Serine     | SER      | 3 SER#P2,3                | [53]       |
| Succinate  | SUC*     | 4 SUC#P1,2; [1]=[4], [2]=[3] | [47] |
| Threonine  | THR      | 4 THR#P2,3,4              | [53]       |
| Valine     | VAL      | 5 VAL#P4,5                | [53]       |

*: metabolite not represented in the reaction network or used in the study

One-dimensional $^1$H-NMR allows for the measurement of positional $^{13}$C-enrichments. The observed positional enrichments are denoted by a leading "P" followed by the position(s). An example for the corresponding measurement model is given in S1 Sec 7.2.
Fig J. Network and $^1$H-NMR labeling measurements. Each measurement group is represented by a circle giving the number of measured fractions.
7.2 Measurement model

Positional enrichments can be expressed – up to a normalization factor – as a linear combination of isotopomer fractions [51], as shown here for the C3 metabolite pyruvate as an example:

\[
P Y R \# P 1 : \begin{pmatrix} P Y R [1] \# 0 \\ P Y R [1] \# 1 \end{pmatrix} = \begin{pmatrix} 1 & 1 & 1 & 1 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 1 & 1 & 1 \end{pmatrix},
\]

where \( x_{PYR} \) denotes the vector of isotopomer fractions of the metabolite PYR. P1 indicates the first C atom position is observed.

7.3 Measurement error model

Up to now, for \(^1\)H-NMR only data-points having a low abundance of \(^{13}\)C labeling content have been published. Errors of observables with high labeling content were linearly extrapolated from these data.

Fig K. Error model for \(^1\)H-NMR based labeling measurements compiled from published data sets (S1 Table F). Standard deviations were determined by linear regression, i.e., by fitting the linear error model \( \sigma = b + m \cdot \eta \) with slope (m) and y-axis intercept (b) to the data resulting in \( \sigma = 0.007267 + 0.004941 \cdot \eta \) (blue line). For data with higher labeling incorporation than 30%, standard deviations were linearly extrapolated (red dotted line).
8. Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS)

8.1 Measurement specification

Table G. GC-C-IRMS measurement group specification.

| Metabolite    | # Carbons | Measurement specification | Reference |
|---------------|-----------|---------------------------|-----------|
| Alanine       | ALA       | 3                         | ALA#M0,1  | [10]      |
| Aspartate     | ASP       | 4                         | ASP#M0,1  | [10]      |
| Glutamate     | GLU       | 6                         | GLU#M0,1  | [10]      |
| Glycine       | GLY       | 2                         | GLY#M0,1  | [10]      |
| Histidine     | HIS       | 6                         | HIS#M0,1  | [10]      |
| Isoleucine    | ILE       | 6                         | ILE#M0,1  | [10]      |
| Leucine       | LEU       | 6                         | LEU#M0,1  | [10]      |
| Phenylalanine | PHE       | 9                         | PHE#M0,1  | [10]      |
| Proline       | PRO       | 5                         | PRO#M0,1  | [10]      |
| Serine        | SER       | 3                         | SER#M0,1  | [10]      |
| Threonine     | THR       | 4                         | THR#M0,1  | [10]      |
| Valine        | VAL       | 5                         | VAL#M0,1  | [10]      |

GC-C-IRMS permits the measurement of the $^{13}$C/$^{12}$C ratio. Typically, measurements are given in $\delta^{13}$C, denoting the $^{13}$C content (expressed in per mill) which is converted into $^{13}$C enrichment. Thus, METAB#M0,1 denotes the content of $^{12}$C to $^{13}$C isotope fractions for a metabolite METAB. An example for the corresponding measurement model is given in S1 Sec 8.2.
Fig L. Network and GC-C-IRMS labeling measurements. Each measurement group is represented by a circle giving the number of measured fractions.
8.2 Measurement model

GC-C-IRMS enrichments can be expressed as a linear combination of isotopomer fractions weighted by the ratio of (un)labeled carbon atoms and total number of carbon atoms, as shown here for the C3 metabolite pyruvate as an example:

\[
\begin{pmatrix}
PYR #0 \\
PYR #1
\end{pmatrix} = \begin{pmatrix}
1 & 2/3 & 1/3 & 2/3 & 1/3 & 2/3 & 1/3 & 1/3 & 0 \\
0 & 1/3 & 1/3 & 2/3 & 1/3 & 2/3 & 2/3 & 1
\end{pmatrix} \cdot \begin{pmatrix}
x_\text{000} \\
x_\text{001} \\
x_\text{010} \\
x_\text{011} \\
x_\text{100} \\
x_\text{101} \\
x_\text{110} \\
x_\text{111}
\end{pmatrix} = \mathbf{M}_{\text{PYR.CIRMS}} \cdot \mathbf{x}_{\text{PYR}}
\]

where \( \mathbf{x}_{\text{PYR}} \) denotes the vector of isotopomer fractions of the metabolite PYR.

8.3 Measurement error model

![Error model for GC-C-IRMS based labeling measurements compiled from published data sets (S1 Table G). Standard deviations were determined by linear regression, i.e., by fitting the linear error model \( \sigma = b + m \cdot \eta \) with slope (m) and y-axis intercept (b) to the data resulting in \(-0.000991 + 0.092765 \cdot \eta \). Since only metabolomics data with a low labeling incorporation were available, instead of this error model a defensive constant error model was chosen, determined by the largest error value: \( \sigma = 0.0231 \).](image-url)

Fig M. Error model for GC-C-IRMS based labeling measurements compiled from published data sets (S1 Table G). Standard deviations were determined by linear regression, i.e., by fitting the linear error model \( \sigma = b + m \cdot \eta \) with slope (m) and y-axis intercept (b) to the data resulting in \(-0.000991 + 0.092765 \cdot \eta \). Since only metabolomics data with a low labeling incorporation were available, instead of this error model a defensive constant error model was chosen, determined by the largest error value: \( \sigma = 0.0231 \).
9. References

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