AIChE
DIVISION 15
Food Pharmaceuticals and Bioengineering

2006 FP&B Student Travel Award Application Form

SCOPE

The AIChE Food, Pharmaceuticals and Bioengineering Division will make available at least five (5) travel awards to graduate students who present posters at the 2006 Annual Meeting in San Francisco. Each travel grant is worth $500.

DEADLINES AND AWARDEE NOTIFICATION

- The completed form and associated documents should be received by August 15, 2006.
- The travel awards will be announced by September 29, 2006.
- Receipts to cover the amount of the award should be submitted after the conference according to instructions that will be provided later to the awardees.
- Notification of an award will be sent to the awardees ONLY.
- Applicants will NOT be notified if not funded.

ELIGIBILITY

- The graduate student should be a member of AIChE Food, Pharmaceuticals and Bioengineering Division.
- The poster(s) must have been accepted and must be presented in one of the Division 15 poster sessions.
- Poster content should not overlap with oral presentations by the same group.
- Award selection will be based on the novelty and quality of the scientific content of the submitted extended abstract.
- The most updated version of the abstract should be uploaded to the conference website before submitting this form and should be attached to this application form.

SUBMISSION INSTRUCTIONS

The completed application form, the extended abstract and a proof of DIVISION 15 and AIChE membership should be e-mailed **as a single PDF file (no larger than 7 Mb)** by August 15, 2006 to FPBE_2006@northwestern.edu. No exceptions to the submission format or time extensions will be considered.

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NOMINEE

Name of Nominee: Avantika Shastri
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By checking this box ☑ I certify that I am a *bona fide* graduate student now and until the meeting time.

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POSTER INFORMATION

Poster Title: Design of Transient Isotopic Labeling Studies for Experimental Measurement of Autotrophic Metabolic Fluxes

Authors: Avantika Shastri and John Morgan

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Design of Transient Isotopic Labeling Studies for the Experimental Measurement of Autotrophic Metabolic Fluxes

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School of Chemical Engineering, Purdue University, W.Lafayette, IN 47907, USA.

1. Introduction

Photosynthesis is an important metabolic process that converts carbon dioxide into organic molecules required for the sustenance of mankind. An immensely wide variety of molecules are synthesized in photosynthetic organisms, including food and important drugs. The knowledge of the interaction of carbon fixation pathways with other metabolic pathways, under different environmental conditions and genetic backgrounds, will prove invaluable in modifying photosynthetic systems as well as increase fundamental biological understanding of photosynthesis.

Metabolic flux analysis (MFA) is an essential component of systems biology, which seeks to quantify the rates of material movement through complex reaction networks. In coordination with the information from proteomic, transcriptomic and genomic studies, metabolic flux maps can help to decipher the phenotypic outcome of genetic and environmental alterations. The determination of metabolic fluxes using ^{13}C tracers is an experimental MFA approach (^{13}C-MFA) that has been the subject of extensive research in the past decade (Yang et al. 2002; Schwender et al. 2004; Sriram et al. 2004). Although many different organisms have been subject to ^{13}C – MFA, all of these have been under conditions of heterotrophic or mixotrophic growth. One of the drawbacks of current ^{13}C-MFA methods is that they can be applied only to systems which feed on substrate molecules containing at least one carbon-carbon bond. The principal information about reaction rates is abstracted from the fact that carbon-carbon bonds of the input are broken and reformed in different ways along different metabolic pathways. Thus, they cannot be applied to photosynthetic systems that grow on carbon dioxide as their sole carbon source.

We are developing a novel *transient* ^{13}C-MFA methodology to measure steady state metabolic fluxes in photosynthetic systems with a single carbon input. Our selected model photosynthetic system, *Synechocystis* sp. PCC 6803, is a fresh water photosynthetic prokaryote, with a fully sequenced genome (Kaneko et al. 1996). It has a simple internal structure compared to plants and algae, while still possessing the same pathways of carbon fixation. It therefore proffers an ideal system to develop ^{13}C MFA tools for photosynthetic organisms. In this work, starting with the isotopomer balancing framework, we identify several critical aspects of transient labeling in a photosynthetic system. Based on this, we have developed a comprehensive plan for the transient ^{13}C MFA studies in *Synechocystis*.

2. Transient ^{13}C – MFA Framework

The detailed modeling framework for steady state ^{13}C MFA has been developed by several competing researchers (Szyperski 1998; Wiechert et al. 2001). A flow chart of a typical steady state ^{13}C – MFA process is shown in Figure 1. In this technique, substrate with carbon labeled at certain positions (^{13}C) is fed to the biological system. Based on the pattern of label incorporation in certain downstream metabolites, indirect information on pathway fluxes is obtained. The labeling patterns are usually detected in the amino acids under metabolic and isotopic steady state conditions, by ^{13}C nuclear magnetic resonance (NMR) or mass spectroscopy (MS) (Wiechert et al. 2001). If the flux map is known, it is possible to simulate the isotopic labeling patterns. The obtained labeling data is therefore used in a constrained non-linear optimization framework to find the flux map that minimizes the difference between predicted and obtained labeling patterns. The steady state mass balances on labeled molecules (isotopomers), the stoichiometric balances and uptake fluxes form the constraints on the problem. The key differences between steady state and transient MFA is the need for transient isotopic labeling data and metabolite pool size data, as shown in Figure 1. These differences guide the development of our method, as described in sections 2.1 – 2.4.

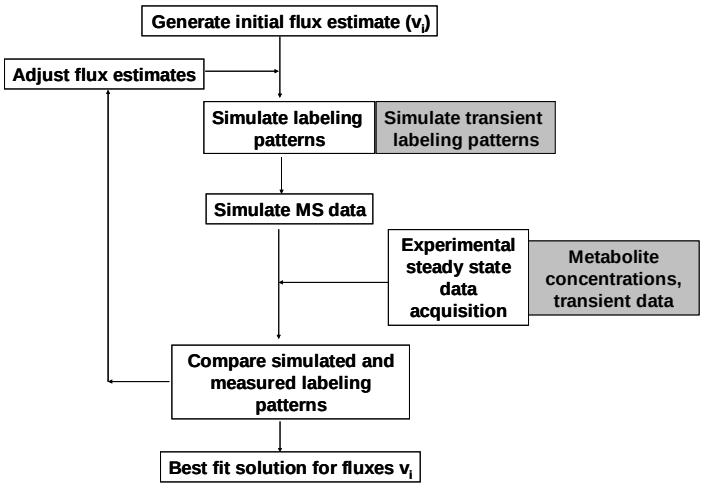

Figure 1. Flowchart showing the overall schematic of ^{13}C-MFA. The grey boxes are the modifications required to apply the process to photosynthetic systems.

2.1. Mathematical Description of Transient Isotopic Balances

The rate of accumulation of label in various isotopomers can be described by a mass balance equation, in matrix-vector form as (Wiechert et al. 1999):

$$\frac{d(\mathbf{CI})}{dt} = \sum_{j=1}^{n} v_j \mathbf{P}_j \mathbf{I} + 0.5 \sum_{j=1}^{n} \mathbf{I}^T v_j \mathbf{Q}_j \mathbf{I} + \sum_{inp=1}^{ninp} v_{inp} \mathbf{P}_{inp} \mathbf{I}_{inp} \qquad (1)$$

where **I** is the overall isotopomer distribution vector, v_j is the flux of the j^{th} metabolic reaction, $\mathbf{P}_j$ is a matrix describing the transitions from reactant to product isotopomers for the j^{th} internal metabolic reaction containing a single reactant, $\mathbf{Q}_j$ is a matrix describing the transitions from reactant to product

isotopomers for the jth internal metabolic reaction involving two reactants and a single product. **C** is a diagonal matrix corresponding to intracellular metabolite concentrations. $\mathbf{P}_{inp}$ is a matrix describing transitions from extra cellular reactants (input) to their intracellular counterparts. $\mathbf{I}_{inp}$ is the isotopomer distribution vector of the input reactants and v_{inp} is the input flux vector. Unlike the case of steady-state labeling methods, where the RHS of equation (1) equals zero, in the transient labeling case, the concentrations of metabolites do not drop out of the equation. Therefore, concentration measurements form a necessary part of the transient isotopic method.

2.2 Optimization framework for Solving Unknown Fluxes

The forward model described in equation (1) can be solved to obtain isotopomer distribution vectors as a function of time, **I**(t), provided the fluxes v_i and metabolite pool sizes are known. Therefore, in order to solve for the fluxes, v_i, an optimization problem is formulated, which minimizes the error between measured labeling patterns and those predicted by equation (1). The differential equations described by equation (1), along with biochemical reaction network stoichiometry (equation (2)) form the constraints of the optimization problem. The formal statement of the problem is described as follows:

Minimize (measured isotopic label – calculated isotopic label)^2 (2)

subject to $S.v = 0$ (3)

$$\frac{d(\mathbf{IC})}{dt} = \sum_{j=1}^{n} v_j \mathbf{P}_j \mathbf{I} + 0.5 \sum_{j=1}^{n} \mathbf{I}^T v_j \mathbf{Q}_j \mathbf{I} + \sum_{inp=1}^{ninp} v_{inp} \mathbf{P}_{inp} \mathbf{I}_{inp} \quad (1)$$

$$v_{bm} = \mu Y \quad (4)$$

$$v_{inp\_co_2} = 100 \quad (5)$$

The basis of the calculation is normalized to the carbon dioxide input, v_{inp_co2} to 100 μmoles per hour, μ is the growth rate (hr^{-1}), and Y the biomass yield per 100 μmoles of CO_2 fixed by the system. Un-measurable pool sizes can also be included as unknown parameters in the problem.

2.3 Choice of Measured Metabolic Intermediates

As stated in section 2.1, concentration measurements form a necessary part of the transient isotopic method. Equation (1) also dictates the choice of internal metabolites most suitable for measurement of labeling patterns and concentrations. Steady state ^{13}C MFA is typically performed by measuring labeling patterns in proteinogenic amino acids. However, in the transient labeling case, in order to restrict the size of the model and number of measured intermediates, the most optimal metabolites to measure are not the amino acids but the central carbon intermediates themselves.

2.4 Metabolite Concentrations and Transient Isotopic Data Acquisition

Steady state MFA procedures utilized both NMR and mass spectroscopy for the determination of labeling patterns. While NMR provides direct information about each labeled ^{13}C-^{13}C bond within a molecule, MS data can only provide partial information about labeled positions. MS methods have much greater sensitivities (several orders of magnitude) than ^{13}C-NMR. This consideration tends to outweigh its drawbacks, and it is increasingly the method of choice in recent developments in the field of ^{13}C-MFA (van Winden et al. 2005; Noh et al. 2006). Transient MFA requires several samples to be taken over a short period of time. The sample size is therefore a critical experimental parameter that affects the practical realization of labeling data acquisition. Therefore, a GC-MS method using BSTFA-10% TMCS-methoxyamine derivatization was selected to measure concentrations of central carbon intermediates (Roessner et al. 2000). Both concentration as well as isotopic labeling data can be obtained by this method.

3. Results

3.1.1 Metabolite Concentration Measurements using GC-MS

The selected GC-MS method (Roessner et al. 2000) was successfully adapted to derivatize, identify and quantify the following metabolites as their trimethylsilyl derivatives: glyoxylate, pyruvate, glycolate, succinate, fumarate, malate, α–ketoglutarate, phosphoenolpyruvate, isocitrate, 3-phosphoglycerate, citrate, erythrose-4-phosphate, ribulose-5-phosphate, fructose-6-phosphate, glucose-6-phosphate, ribulose-1,5- bisphosphate.

3.1.2 Extraction and Quantification of Metabolites from *Synechocystis* Cultures

Shake flask cultures of *Synechocystis* cells were grown autotrophically in shake flasks till late log phase (Shastri and Morgan 2005). The cells were quenched with equal volumes of a 60% methanol water mixture at -40 ^{o}C. The cells were then centrifuged at 8000 g for 15 minutes, at -20 ^{o}C, and the pellets were used for further extraction. Each sample corresponded to approximately 7.5mg dry cell mass.

Figure 2 shows GC-MS chromatograms of the methanol extracts (1 + 2) of *Synechocystis*. A third extract made on the pellet, showed no identifiable metabolites in the GC chromatogram, using characteristic ions in SIM mode (Figure 3). We conclude that two extractions are sufficient to quantitatively extract all the metabolites from the cell pellet.

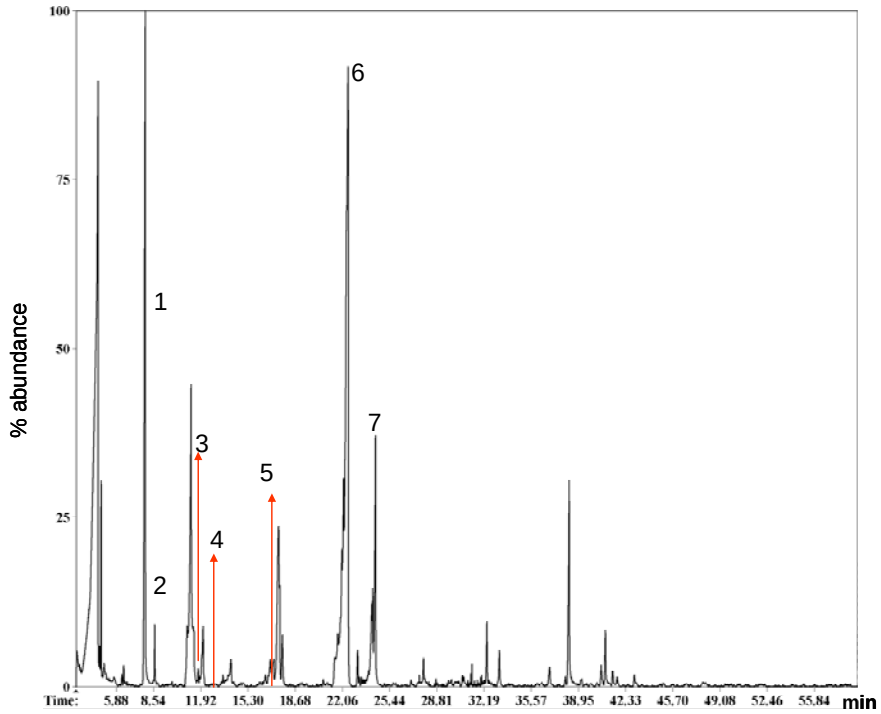

Figure 2. Chromatogram showing the total ion current (TIC) for combined extract 1 and 2, using 7.5 mg (dry wt) *Synechocystis*. Most of the highly abundant peaks are unknowns. Only a few of the identified metabolites have been labeled. The peaks are labeled as follows: 1 and 2 = derivatization artifacts, 3 = succinate, 4 = fumarate, 5 = malate, 6 = ribitiol (internal standard), 7 = isocitrate.

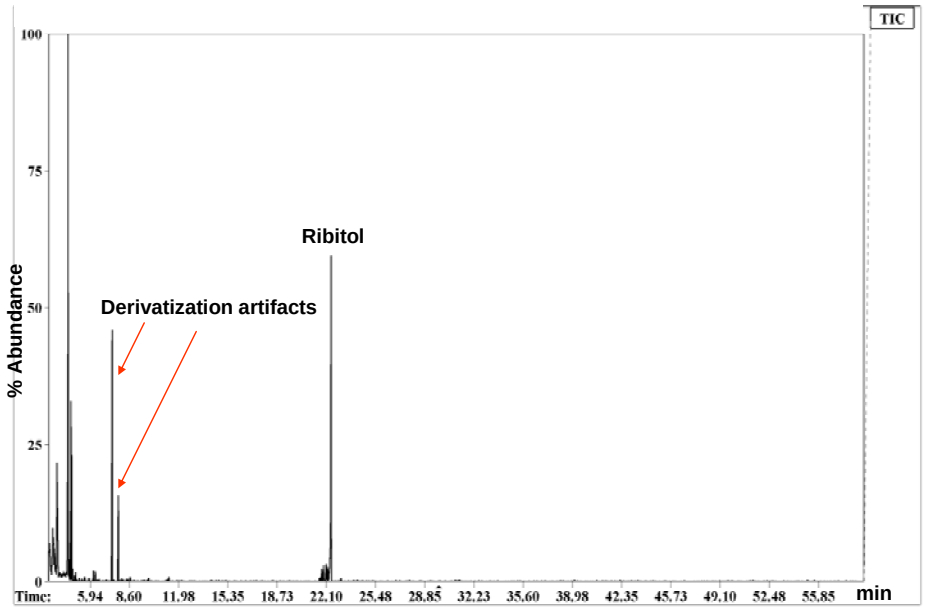

Figure 3. Chromatogram of the third extraction of 7.5 mg (dry wt) *Synechocystis*. No metabolite of interest was detected. TIC = total ion current.

3.2 Simulation of Transient Isotopomer Labeling

The central metabolic pathways of *Synechocystis* were annotated from its genomic data (Kaneko et al. 1996). The reconstructed reaction network consists of 76 reactions, and involves 37 metabolites. Reversible reactions were divided into two separate fluxes: forward and backward. Reactions were defined to be of three types, uni (one reactant, one product), uni-bi (one reactant, two products), and bi-uni (two reactants, one product). Reactions of the type bi-bi (two reactants, two products) were split into two reactions, a bi-uni and a uni-bi, with a fictitious unimolecular intermediate. The biomass formation equation was formulated based on biomass composition measurements made on mid-exponential phase batch cultures of *Synechocystis* (Shastri and Morgan 2005). A flux distribution was estimated from our previous work, using linear programming to maximize biomass flux, while minimizing light utilization (Shastri and Morgan 2005) .

Using the reconstructed network, flux estimates and pool sizes, equation (1) was solved numerically using the ode23 solver in MATLAB v. 7.01 (MathWorks, Natick, MA). Labeling patterns were simulated over a period of 10 minutes for two different input labeling scenarios, a step change to 100% ^{13}C, and a step change to 30% ^{13}C. Figure 4 and Figure 5 show the mass distribution vectors (MDVs) for selected metabolites for the two cases. For the 100% step change, we see a 100 % shift from the completely unlabeled to the fully labeled molecules for all the metabolites, while traversing through all intermediate mass fractions, as can be expected. Metabolite pools closer to the carbon fixation reactions get labeled faster than molecules far downstream (3phosphoglycerate versus α-ketoglutarate). The observed transients are more dramatic for the 100% step change, with each mass fraction of each metabolite going through a maximum. This makes the intensity of each mass fraction reach a significant (measurable) level at some point of time during the experiment. For the 30% labeling case, the higher mass fractions do not reach very high concentrations, and may not be measurable by MS. The optimal labeling strategy will therefore involve utilization of as high a step change as is practically feasible.

The simulation of the forward model was performed under a number of assumptions as stated in the beginning of this section. The flux distribution used did not contain any reversible fluxes, futile cycles. These shortcomings are inherent to the linear programming approach used to estimate the fluxes (Shastri and Morgan 2005). While the action of reversible fluxes will serve to slow the labeling dynamics by back mixing, fluxes which are actually much larger than the estimates will serve to decrease the labeling time constants. If all critical fluxes are an order of magnitude larger than the estimated fluxes, the timescale of the entire labeling process will decrease by an order of magnitude (one minute instead of 10 minutes). Despite these potential pitfalls, the simulations still provide a reasonable estimate for the labeling timescale that can be used for a first ^{13}C labeling experiment.

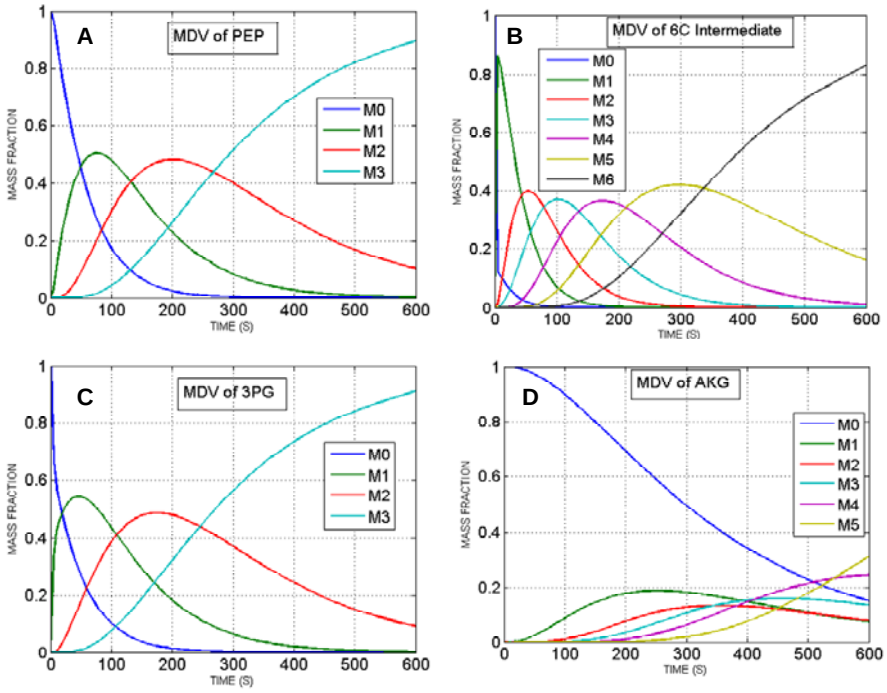

Figure 4. Mass distribution vectors (MDV) of selected intermediates in response to a carbon dioxide step change from 0 – 100% ^{13}C. PEP = phosphoenol pyruvate, 3PG = 3 phosphoglycerate, AKG = α-ketoglutarate, MDV = mass distribution vector.

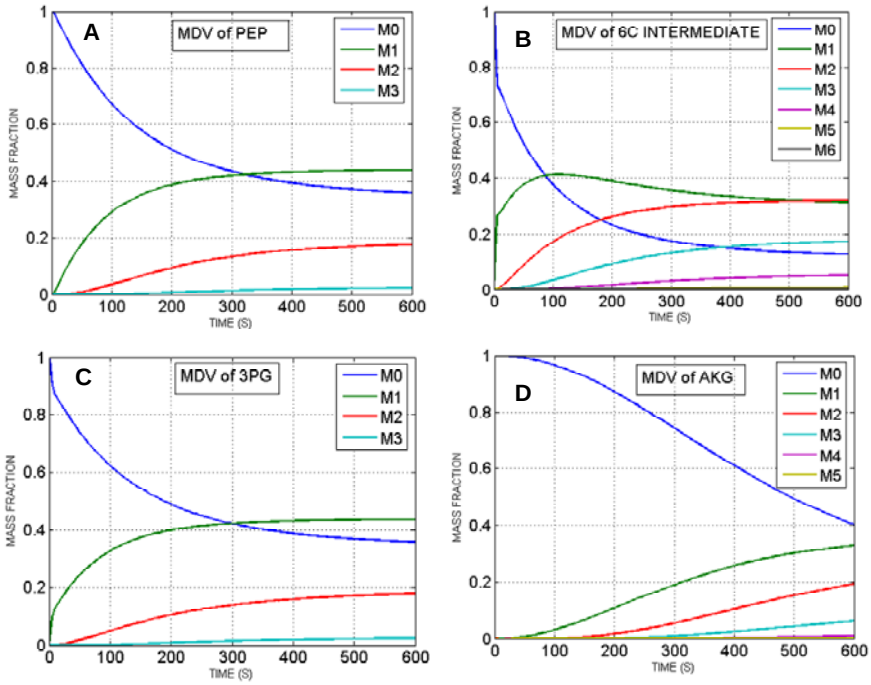

Figure 5. Mass distribution vectors (MDV) of selected intermediates in response to a carbon dioxide step change from 0–30% ^{13}C. PEP = phosphoenol pyruvate, 3PG = 3 phosphoglycerate, AKG = α-ketoglutarate, MDV = mass distribution vector.

4. Conclusions and Future Work

A novel methodology to measure steady state photosynthetic fluxes using transient ^{13}C isotopic labeling was outlined. Based on the overall framework of ^{13}C-MFA, critical factors influencing the transient method, as compared to previous steady state methods, were identified. These included the choice of labeled metabolites to measure, and the need for metabolite pool size measurements. A GC-MS method, in tandem with suitable quenching and extraction procedures, was used to measure several key metabolites in *Synechocystis*. The transient isotopic label balances, along with measured metabolite pool sizes and simulated flux distributions, were used to simulate labeling patterns. The simulations indicate a time frame of 10 minutes in which the labeling transients occur in central carbon metabolism. These results guide our future work in experimentally obtaining labeling patterns rapidly within 10 minutes. The GC-MS technique selected is suitable for both concentration and isotopic measurements. Future work includes obtaining transient isotopic data as guided by this work, and development of a suitable optimization framework to obtain the final optimum flux distribution.

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