

Estimation of Bidirectional Metabolic Fluxes from MS and NMR Data Using Positional Representations

Marcos J. Araúzo-Bravo

marara@dali.eis.uva.es

Kazuyuki Shimizu

shimi@bse.kyutech.ac.jp

Department of Biochemical Engineering and Science, Kyushu Institute of Technology,
Iizuka, Fukuoka 820-8502, Japan

Abstract

It is quite important to estimate the metabolic flux distribution (MFD) vectors *in vivo*, and to investigate the effect of culture environments on the flux distributions to uncover the metabolic regulation mechanism of microbial cells. The conventional approach is to compute the MFD using the stoichiometric equations and the measured specific rates (input and output variables). However, this method cannot give the MFD for the complex metabolic network which includes cyclic pathways. In the present investigation, we considered the method of analysing the metabolic fluxes based on ^{13}C tracer experiments. In particular, we compared the different techniques of estimating the bidirectional fluxes in the metabolic networks, studying their applicability with respect to the different types of data formats obtained through GC-MS (gas chromatography-mass spectrometry) and NMR (nuclear magnetic resonance) measurements in labeling experiments. It was found that some techniques cannot be applied for GC-MS and NMR data. In the present research, therefore, a new preprocessing method for MS and NMR data was developed, to solve some of the problems encountered in the conventional approaches.

Keywords: Metabolic flux analysis (MFA), bidirectional metabolic fluxes, isotopic tracer, mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy, positional enrichment

1 Introduction

Metabolic fluxes constitute a fundamental tool in determining the cell physiology, providing a measure of the degree of engagement of various pathways in cellular functions and metabolic processes. Therefore, an accurate quantification of the magnitude of pathway fluxes *in vivo* is an important goal for metabolic engineering. The aim of the metabolic flux analysis is to calculate the unmeasurable metabolic fluxes from the measured quantities and the stoichiometric equations. The conventional approach to do this is to apply mass balances with stoichiometric equations. However, this method cannot compute the metabolic flux distribution (MFD) for the complex systems which include cyclic pathways such as TCA cycle or pentose phosphate pathway. An alternative method to overcome this problem is to make the isotope balance using the measurements data of NMR and/or GC-MS. Several analysis methods have been proposed to compute MFD using isotopomer distributions [3, 6, 10]. However, the computational burden is demanding and the analysis methods are still under much argue. In the present article, a brief review is made on several approaches and then a new preprocessing method is proposed.

2 Types of Data from Labeling Experiments

Carbon isotope labeling studies fall within the category of tracer experiments. There are several ways of representing the information generated from such experiments:

- **Positional ^{13}C labeling:** Gives information about the concentration of labeled carbon in each position of the atom carbon backbone. The important definitions for positional labeling are as follows:
 - *Positional enrichment (PE)* [10]: The *positional enrichment* at the i th carbon atom within a metabolite M is the sum of all the *isotopomer fractions* of M, where the i th carbon atom is labeled [10]. This concept is equivalent to the concept of *fraction labeling* used in [2].
 - *Metabolic activity vector (MAV)* [13]: The vector of all the positional enrichment of a metabolite. Its n th element contains the specific enrichment of the n th carbon atom in the corresponding molecule [14].
 - *Mass distribution vector (MDV)* [13]: This may be placed between *MAV* and *IDV (Isotopomer Distribution Vectors)* with respect to its complexity. Assuming an organic molecule with n carbon atoms that can occur as ^{12}C or ^{13}C , $n+1$ different masses are obtained, ranging from the lowest mass ^{12}C -isotopomer to the highest mass ^{13}C -isotopomer. They contain the molar fraction of a group of isotopomers with the same mass in every element.
- **Isotopomer:** The MS and NMR measurements in labeled experiments give the concentrations of some vectors formed by labeled and unlabeled carbon atoms. These vectors are known as *isotopomers*, since these labeling patterns can be interpreted as *isotope isomers* [6]. The important definitions for isotopomer are as follows:
 - *Isotopomer* [10]: Considering only the ^{12}C and ^{13}C isotopes in the carbon backbone of a molecule M having n carbon atoms, an *isotopomer* of M is one of the 2^n possible labeling states.
 - *Isotopomer fraction* [10]: Denotes the percentage of molecules in a specific labeling state. An important difference with respect to the positional fractions is that the isotopomer fractions always add up to 100%, whereas positional labeling fractions have no such constraint.
 - *Isotopomer Distribution Vectors (IDV)* [6, 13]: Contain the mole fraction of individual isotopomers.
 - *Cumomer fraction* [10]: This word is an abbreviation of “*cumulated isotopomer*” and means a certain sum of isotopomer fractions of a metabolite. They allow to solve analytically the nonlinearity and high dimensionality of the isotopomer balance equations.

For better understanding of the relations among those representations, Fig. 1 gives the relation between *IDV* and *MAV* for a molecule of alanine (with three carbons). The columns show the eight isotopomers that constitute the *IDV*, and the estimated isotopomer fraction is given at the top of each column, while each row shows all the atoms that contribute to generate the *MAV*, and the three corresponding positional enrichment values are given at the rightmost position. It is also shown that the *IDV* elements sum up to 1, while the *MAV* elements do not necessarily give 1.

3 States and Transitions Representations

In order to formulate the bidirectional flux analysis, it is necessary to establish the state variables of the system, fluxes and metabolites states, and to define the transitions between these states. Several representations have been proposed in the literature for these elements. If the metabolic flux for the i th

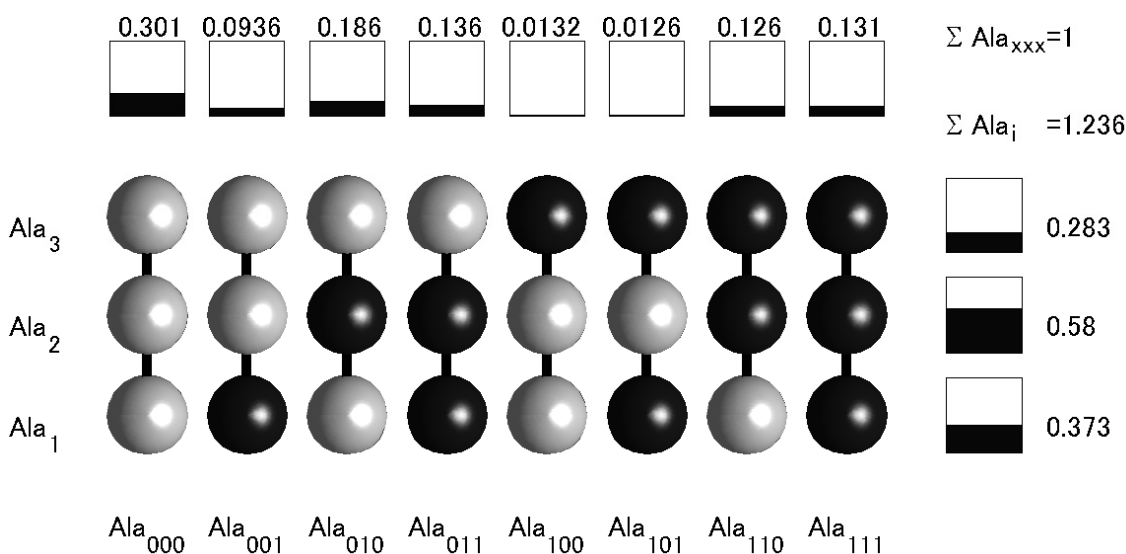


Figure 1: Relation between *IDV* and *MAV* for the numerical example of the alanine. All the isotopomers that constitute the *IDV* are shown in columns. The estimated isotopomer fractions are given at the top of each column, while each row shows all the atoms that contribute to generate the *MAV*. The corresponding positional enrichment is given at the rightmost side.

reaction is expressed by v_i , a distinction is made between both forward and backward directions using the symbols v_i^{\rightarrow} and v_i^{\leftarrow} . These fluxes constitute the natural flux coordinates [9]. The stoichiometric balance allows only the determination of net fluxes such that $v_i^{net} = v_i^{\rightarrow} - v_i^{\leftarrow}$, but not the forward and backward fluxes. For practical interpretation of the flux values and for numerical reasons the direct use of these fluxes variables is not appropriate. To obtain a more suitable representation of the bidirectional steps, *net fluxes*, v_i^{net} , and *exchange flux*, v_i^{xch} have been proposed in [9] such that

$$v_i^{net} = v_i^{\rightarrow} - v_i^{\leftarrow}, \quad v_i^{xch} = \min(v_i^{\rightarrow}, v_i^{\leftarrow}) \quad (1)$$

which are denominated to be application flux coordinates. Finally, to make the treatment of the infinite exchange values associated with rapid equilibrium reactions numerically feasible, a rescaling to a bounded range [0 1] is made using a compacting operation such that $v_i^{res} = \frac{v_i^{xch}}{\beta + v_i^{xch}}$. The parameter β is chosen on the order of the magnitude of the system input flux [9]. The rescaled exchange flux v_i^{res} within the net flux v_i^{net} constitutes the numerical flux coordinates. The transformation equations between these coordinates systems, and the main features of the latter are shown in Table 1.

In the case of labeling experiments, the information provided for a metabolite with n carbon labeled backbone may be classified based on the two different labeled metabolite representation levels:

- **Positional level**, which represents the concentrations of the labeled carbon in each of the n positions of the carbon backbone of each metabolite.
- **Isotopomer level**, which represents the normalized concentrations of each of the 2^n isotopomers of the carbon backbone of each metabolite.

The labeling representation levels show the detailed level in which the labeled information is taken into account. The representations at positional level are simpler than at isotopomer level. In

Table 1: Transformation equations between the flux coordinates systems. All flux coordinates v_i^{\rightarrow} , v_i^{\leftarrow} , v_i^{net} and v_i^{xch} have the same dimension and physical unit, while v_i^{res} are dimensionless.

Natural flux coordinates	Forward and backward fluxes v_i^{\rightarrow} , v_i^{\leftarrow}
Application flux coordinates	Net and exchange fluxes v_i^{net} , v_i^{xch}
Makes easy to express irreversibility ($v_i^{xch} = 0$) and rapid equilibrium ($v_i^{xch} = \infty$)	
Using the transformation:	$\Phi : \begin{pmatrix} v_i^{net} \\ v_i^{xch} \end{pmatrix} \rightarrow \begin{pmatrix} v_i^{\rightarrow} \\ v_i^{\leftarrow} \end{pmatrix} = \begin{pmatrix} v_i^{xch} - \min(-v_i^{net}, 0) \\ v_i^{xch} - \min(v_i^{net}, 0) \end{pmatrix}$
and its inverse:	$\Phi^{-1} : \begin{pmatrix} v_i^{\rightarrow} \\ v_i^{\leftarrow} \end{pmatrix} \rightarrow \begin{pmatrix} v_i^{net} \\ v_i^{xch} \end{pmatrix} = \begin{pmatrix} v_i^{\rightarrow} - v_i^{\leftarrow} \\ \min(v_i^{\rightarrow}, v_i^{\leftarrow}) \end{pmatrix}$
Numerical flux coordinates	Net and [0 1]-rescaled exchange fluxes v_i^{net} , v_i^{res}
Makes the treatment of infinite values associated with rapid equilibrium ($v_i^{res} = 1$) numerically feasible	
Using the transformation:	$\Phi_{\beta} : \begin{pmatrix} v_i^{net} \\ v_i^{res} \end{pmatrix} \rightarrow \begin{pmatrix} v_i^{net} \\ v_i^{xch} \end{pmatrix} = \begin{pmatrix} v_i^{net} \\ \beta \cdot \frac{v_i^{res}}{1-v_i^{res}} \end{pmatrix}$
and its inverse:	$\Phi_{\beta}^{-1} : v_i^{xch} \rightarrow v_i^{res} = \frac{v_i^{xch}}{\beta + v_i^{xch}}$

practice, the positional representation cannot be used directly for data obtained from MS and NMR measurements. For this reason, a new numerical processing technique for MS and NMR data has been developed to allow its use with the simplest methods of the positional representation. Moreover, to store the information about the fate of the labeled carbons across the network and to decouple the generation of the steady state equations from the details of the transfer of carbon atoms from reactants and products, two types of transfer state matrices can be chosen: mapping matrices and transition matrices. The two types give equivalent information, but the size of the mapping matrices includes only information about the fates between the substrate and the product metabolite, and the size of transition matrices includes information about all possible metabolites, being much larger but with a fixed structure. Note that all of the mapping matrices are the same size.

The several transfer matrices are associated to balances at different representation levels. Consider the following biochemical reaction



where A , B , C and D are metabolite activity vectors. It is possible to formulate the following balances:

- **Stoichiometric mass balance:** In the molecular representation level, the metabolic concentration of an intracellular metabolite pool must add up to zero in the stationary state due to mass balance. This gives linear metabolic flux balances equations

$$A + B - C - D = 0 \quad (3)$$

- **Isotope balance [14]:** In the positional level, the insertion of the atom mapping matrices: $AMM_{A>C}$, $AMM_{B>C}$, $AMM_{A>D}$ and $AMM_{B>D}$, and metabolite activity vectors \mathbf{A} , \mathbf{B} , \mathbf{C} and \mathbf{D} transform the mass balance equations into an isotope balance

$$(AMM_{A>C} \cdot \mathbf{A}) + (AMM_{B>C} \cdot \mathbf{B}) = \mathbf{C}, \quad (AMM_{A>D} \cdot \mathbf{A}) + (AMM_{B>D} \cdot \mathbf{B}) = \mathbf{D} \quad (4)$$

- **Isotopomer balance [7]:** In the isotopomer level, the balance is made by substituting the atom mapping matrices (AMM) with the isotopomer mapping matrices (IMM), the metabolite

activity vectors with isotopomer distribution vectors \mathbf{I}_A , \mathbf{I}_B , \mathbf{I}_C and \mathbf{I}_D , and the addition operator $+$ with the elementwise multiplication operator \otimes :

$$(IMM_{A>C} \cdot \mathbf{I}_A) \otimes (IMM_{B>C} \cdot \mathbf{I}_B) = \mathbf{I}_C, \quad (IMM_{A>D} \cdot \mathbf{I}_A) \otimes (IMM_{B>D} \cdot \mathbf{I}_B) = \mathbf{I}_D \quad (5)$$

It should be noted that the simplest representation level (the molecular level) does not allow bidirectional flux estimation and that the positional and isotoper levels allow it. For two metabolites A and C with n and m carbons respectively, the isotope mapping matrix $IMM_{A>C}$ is a $2^n \times 2^m$ matrix and the atom mapping matrix $AMM_{A>C}$ is a simpler $n \times m$ matrix. For this reason it is interesting to develop a method to use positional representation to solve the bidirectional flux estimation problem.

4 General Approach to the Bidirectional Fluxes Estimation

Based on the choice of the metabolite labeled representation level and of the transfer matrices, it is possible to classify the relevant methods for the estimation of the bidirectional fluxes as shown in Table 2. It is useful to establish a general approach that comprises all the approaches based on the following steps:

- **Preparation:**

1. Obtain the labeled data.
2. Data preprocessing. Correct natural isotopes and obtain metabolic activity vectors in the case of using methods at positional representation level.
3. Assume the biochemical pathways.

- **Establish the general flux model equations:** This model allows the formulation of the activities of the metabolites as functions of the fluxes. In the case of transition matrices this model is a linear system with a square matrix.

1. Introduce the state variables: fluxes and metabolites activities at positional or at isotopomer representation level.
2. Construct the transfer matrices (mapping or transition matrices).
3. Write the stoichiometric equations either at positional or at isotopomer representation level.
4. Obtain the **general flux model equations**: Using the transfer matrices to express the stoichiometric equations in the representation level of the method (positional or isotopomers).
5. Formulate linear constraints (equality and inequality constraints).
6. Choose the free fluxes: These are a set of independent fluxes that fixes the remaining linear degrees of freedom of the constraints. Its values are searched by an optimization method. Once known, they allow to obtain the remaining fluxes through inverting the constraints equations.

- **Estimation of the free fluxes** (and all the state variables): Using an iterative technique,

1. Initialize a set of free fluxes.
2. Obtain the metabolites activities by solving the **general flux model equations** as a function of the free fluxes. In the case of mapping matrices use Gauss-Seidel techniques, while in the case of transition matrices, invert the simulation activity matrix.
3. Compare the state estimates with the fluxes and metabolite activities measurements. Obtain the error between the estimations and the measurements.

Table 2: Classification of the estimation methods for bidirectional fluxes based on the metabolite labeled representation level and on the transfer matrices.

<u>Representation level</u> Transfer matrices	Positional	Isotopomer
Mapping matrices	Zupke and Stephanopoulos [14]	Schmidt <i>et al.</i> [6, 7, 1]
Transition matrices	Wietchert <i>et al.</i> [3, 9, 12]	Wietchert <i>et al.</i> [4, 10, 11]

- If it is more than a threshold, search a new set free fluxes and go to step 2.
 - If it is less than a threshold, finish the search of free fluxes.
4. Obtain the application fluxes.
 5. Obtain the natural fluxes.

5 Method to Estimate Positional Representation Vectors from MS and NMR Data

The representations at atomic level are simpler, require less memory, than the representations at isotopomer level, since for a metabolite of n carbons the second representation needs a vector of 2^n components while the first needs only n . With the idea that the simpler the better, the best representation to solve the problem of metabolic flux analysis seems to be the positional representation. Unfortunately, this representation can not be obtained in practice from MS and NMR data because these techniques generate information at the isotopomer level and not at the positional level. For every metabolite with n carbons, MS generates $n + 1$ data corresponding to the sum of the respective isotopomers with $0, \dots, n$ labeled carbons. The NMR generates some linear combination of individual isotopomers, but not all of them.

If all the $2^n - 1$ labeled isotopomers of an n carbon metabolite are known (it is not necessary to know the unlabeled isotopomer $IDV_{0\dots 0}$, since the sum of all the isotopomers is one), it is possible to obtain n positional enrichment components (that constitute the metabolic activity vector). Unfortunately, the MS and NMR measurements generally do not generate all the isotopomers, making it impossible to apply the methods based on positional representation level techniques. In these cases the Metabolic Flux Analysis techniques are applied [6, 7, 10, 4] using the most complex representations at isotoper level.

In order to estimate bidirectional metabolic fluxes with methods based on positional enrichment representations, it is necessary to compare positional enrichment simulation with positional enrichment measurements. But the positional enrichment measurements cannot be obtained directly. Here a method for their estimation based on MS and NMR measurements was implemented, including the following steps:

1. **Isotopomer estimation:** Estimate all the isotopomers from the partial information from the MS and NMR measurements. This estimation is based on deriving a linear system from the MS and NMR data using a method inspired by [5]:

$$A \cdot IDV^{-0\dots 0} = B, \quad \text{where } A = \begin{pmatrix} A_{MS} \\ A_{NMR} \end{pmatrix}, \quad B = \begin{pmatrix} B_{MS} \\ 0 \end{pmatrix}, \quad (6)$$

and $IDV^{-0\dots 0}$ is the isotopomer distribution vector resulting from the elimination of the unlabeled isotopomer $IDV_{0\dots 0}$ from the former isotopomer distribution vector IDV , and A_{MS} , A_{NMR} and B_{MS} contain the structural information that relates the MS and the NMR signals (singlet, doublets and doublet of doublet [8]) to the respective isotopomers:

- A_{MS} submatrix: Matrix of zeros and ones, storing information about which isotopomers contribute to each MS signal. It has $2^n - 1$ columns and as many rows as MS measurements (n , not considering the unlabeled isotopomer $IDV_{0\dots 0}$, which is a complement to 1 of the sum of the labeled isotopomers).
- B_{MS} subvector: Vector of the MS measurement signals, not including the unlabeled measurement MDV_m .
- A_{NMR} submatrix: Its rows contain the structural information that relates the singlet, doublets and doublet of doublet NMR signals with the respective isotopomers. They are built, considering which are the isotopomers that contribute to each signal. For example, consider the case of a linear chain of carbon atoms.
 - *Singlet signals*: The set of isotopomers that contribute to a singlet signal S in the i th carbon is $Set_{iS} = \{IDV_{\dots x01_i 0x\dots}\}$, where $x = \{0, 1\}$, “ \dots ” means an undefined series of x and 1_i means that the i th position is labeled.
 - *Doublet - signals*: The set of isotopomers that contribute to a doublet- signal $D-$ in the i th carbon is $Set_{iD-} = \{IDV_{\dots x01_i 1x\dots}\}$.
 - *Doublet + signals*: The set of isotopomers that contribute to a doublet+ signal $D+$ in the i th carbon is $Set_{iD+} = \{IDV_{\dots x11_i 0x\dots}\}$.
 - *Doublet of doublet signals*: The set of isotopomers that contribute to a doublet of doublet signal DD in the i th carbon is $Set_{iDD} = \{IDV_{\dots x11_i 1x\dots}\}$.

In the case of branched molecules, aromatic rings or long range couplings between distant carbon atoms, the same ideas can be applied (with much more complex notation), considering the corresponding neighbourhood relations between the carbon atoms. Once these sets are obtained, for each signal without singlets $NS = \{D+, D-, DD\}$, it is possible to add a row in the matrix A_{NMR} , that gives the following equation:

$$Set_{iS} \cdot IDV_{iS} - Set_{iNS} \cdot IDV_{iNS} = 0 \quad (7)$$

where IDV_{iS} and IDV_{iNS} are the intensities of the singlet and signals without singlets respectively, associated with the i th carbon of the metabolite.

The linear system (6), that generally is not square, is solved using a linear least-square method with non-negative constraints. The solution is determined using the pseudo-inverse matrix A , so that $IDV^{-0\dots 0} = (A^T A)^{-1} A^T B$. The sensitivity of the solution to errors in measurements is verified through determining the condition number (ratio of the largest eigenvalue divided by the smallest one). High condition numbers (> 100) indicate that there are redundant equations [5]. Those can be eliminated by an iterative process that eliminates one equation at each step and recomputes the condition number.

2. **Metabolic activity vector calculation:** The metabolic activity vector MAV is obtained through multiplication of the transformation matrix $IDV2MAV$ by $IDV^{-0\dots 0}$:

$$MAV = IDV2MAV \cdot IDV^{-0\dots 0} \quad (8)$$

where the matrix $IDV2MAV$ is a matrix of zeros and ones, distributed in n rows and $2^n - 1$ columns, and built with the consideration that the j th column of the i th row is one, if and only if the i th digit of the binary representation of the j th isotopomer is one. This means that all the isotopomers that have labeled the i th carbon contribute for the i th positional enrichment.

Table 3: Synthetic MS (left) and NMR (right) values for MAV estimation of alanine.

Frag.	m+0	m+1	m+2	m+3	Carbon No.	S	D-	D+	DD	
Ala	123	0.301	0.293	0.275	0.131	2	0.19	0.26	0.28	0.27
						3	0.83	0.17	-	-

Since this algorithm gives all the positional information of *MAV*, it is possible to use directly MS and NMR measurements for estimating the bidirectional metabolic fluxes with positional representation methods.

6 A Simple Example for MS and NMR Data Preprocessing

To illustrate the preprocessing method proposed here, assume that the MS and NMR measurements are known for alanine. They will be used to estimate the positional enrichment of this amino acid. The knowledge of the positional enrichments of the amino acids generated in a biochemical network is especially important since the amino acids are synthesized by the unique pathways from precursor metabolites. Then the positional enrichments of their individual carbons directly reflect the positional enrichments in the precursor, thus being useful to estimate the latter, giving in this way the preprocess measured information for the metabolite activities, needed in step 3 of the free fluxes estimation phase (Section 4). First, it is necessary to estimate all the isotopomers from structural information

$$\begin{pmatrix} 1 & 1 & 0 & 1 & 0 & 0 & 0 \\ 0 & 0 & 1 & 0 & 1 & 1 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 1 \\ \hline 0 & Ala_{2S} & -Ala_{2D-} & 0 & 0 & 0 & 0 \\ 0 & Ala_{2S} & 0 & 0 & 0 & -Ala_{2D+} & 0 \\ 0 & Ala_{2S} & 0 & 0 & 0 & 0 & -Ala_{2DD} \\ 0 & 0 & 0 & Ala_{3S} & Ala_{3S} & -Ala_{3D-} & 0 \end{pmatrix} \cdot \begin{pmatrix} Ala_{001} \\ Ala_{010} \\ Ala_{011} \\ Ala_{100} \\ Ala_{101} \\ Ala_{110} \\ Ala_{111} \end{pmatrix} = \begin{pmatrix} Ala_{m+1} \\ Ala_{m+2} \\ Ala_{m+3} \\ 0 \\ 0 \\ 0 \\ 0 \end{pmatrix} \quad (9)$$

where Ala_{iSig} is the relative intensity of the NMR signal $Sig = \{S, D-, D+, DD\}$ of the i th carbon of alanine where $i = \{2, 3\}$, Ala_{xxx} is the concentration of the xxx isotopomer with $x = \{0, 1\}$, and Ala_{m+i} is the mass spectrometry signal produced by the alanine molecule of molecular weight of $m+i$ where $i = \{1, 2, 3\}$ and m is the molecular weight of the non labeled alanine molecule.

For the example data of Table 3, the condition number of the matrix associated with equation (9) is 41.36 (< 100), which indicates that all the equations are considered to be independent, and the isotopomers vectors can be obtained by inverting the matrix of the system. However, in general, it is necessary to apply a linear optimization technique with positive constraints. The estimated *IDV* is shown in the top line of Fig. 1 (the unlabeled isotopomer Ala_{000} was obtained using the condition that the sum of all the isotopomers is unity). The *MAV* is obtained from the *IDV* using the following *IDV2MAV* transformation matrix:

$$\begin{pmatrix} Ala_1 \\ Ala_2 \\ Ala_3 \end{pmatrix} = \begin{pmatrix} 1 & 0 & 1 & 0 & 1 & 0 & 1 \\ 0 & 1 & 1 & 0 & 0 & 1 & 1 \\ 0 & 0 & 0 & 1 & 1 & 1 & 1 \end{pmatrix} \cdot \begin{pmatrix} Ala_{001} \\ Ala_{010} \\ Ala_{011} \\ Ala_{100} \\ Ala_{101} \\ Ala_{110} \\ Ala_{111} \end{pmatrix} \quad (10)$$

where Ala_i is the positional enrichment of the carbon i of alanine where $i = \{1, 2, 3\}$. These values are shown in the rightmost of Fig. 1.

7 Conclusion and Discussion

The techniques for the estimation of the bidirectional fluxes reported in the literature were classified based on the concept of positional and isotopomer representation levels and the transfer matrices. The representation level concept was used to show why the simplest positional methods cannot be used for MS and NMR data. To overcome this problem, an algorithm was proposed here where all the isotopomers were obtained from MS and NMR measurements and then the information was transformed into positional enrichment. It is important to note that the proposed method is limited to MS and NMR measurements of metabolites with reduced number of carbon atoms, since the first stage of preprocessing: estimation of the isotopomers may give meaningless results for metabolites with more than 5 carbons. It should be noted that even though the isotopomer representation methods do not require the proposed transformation they also have the dimensional problem, since these representations require state vectors of order 2^n and the mathematical effort becomes very high, more than 1000-dimensional nonlinear equation system must be solved repeatedly in a normal biochemical network [11]. For this reason, in such methods molecules with a large number of carbon atoms should be avoided as suggested in [11].

References

- [1] Christensen, B. and Nielsen, J., Isotopomer analysis using GC-MS, *Metabolic Engineering*, 1:282–290, 1999.
- [2] Christensen, B. and Nielsen, J., Metabolic network analysis of *Penicillium chrysogenum* using ^{13}C -labeled glucose, *Biotechnology and Bioengineering*, 68(6):652–659, 2000.
- [3] Marx, A., De Graaf, A.A., Wiechert, W., Eggeling, L., and Sahm, H., Determination of the fluxes in the central metabolism of *Corynebacterium glutamicum* by nuclear magnetic resonance spectroscopy combined with metabolic balance, *Biotechnology and Bioengineering*, 49(2):111–129, 1996.
- [4] Möllney, M., Wiechert, W., Kownatzki, D., and De Graaf, A.A., Bidirectional reaction steps in metabolic networks IV: Optimal design of isotopomer labeling systems, *Biotechnology and Bioengineering*, 66(2):86–103, 1999.
- [5] Noronha, A.B., Yeh, H.J.C., Spande, T.F., and Shiloach, J., Investigation of the TCA cycle and the glyoxylate shunt in *Escherichia coli* BL21 and JM109 using ^{13}C -NMR/MS, *Biotechnology and Bioengineering*, 68(3):316–327, 2000.
- [6] Schmidt, K., Carlsen, M., Nielsen, J., and Villadsen, J., Modeling isotopomer distributions in biochemical networks using isotoper mapping matrices, *Biotechnology and Bioengineering*, 55(6):831–840, 1997.
- [7] Schmidt, K., Nielsen, J., and Villadsen, J., Quantitative analysis of metabolic fluxes in *Escherichia coli* using two dimensional NMR spectroscopy and complete isotoper models, *Journal of Biotechnology*, 71:175–190, 1999.
- [8] Szyperski, T., Biosynthetically directed fractional ^{13}C -labeling of proteinogenic amino acids. An efficient analytical tool to investigate intermediary metabolism, *European Journal of Biochemistry*, 232:433–448, 1995.

- [9] Wiechert, W. and De Graaf, A.A., Bidirectional reaction steps in metabolic networks I: Modeling and simulation of carbon isotope labeling experiments, *Biotechnology and Bioengineering*, 55(1):101–117, 1997.
- [10] Wiechert, W., Möllney, M., Iserman, N., Wurzel, M., and De Graaf, A.A., Bidirectional reaction steps in metabolic networks III: Explicit solution and analysis of isotopomer labeling systems, *Biotechnology and Bioengineering*, 66(2):69–85, 1999.
- [11] Wiechert, W., Möllney, M., Petersen, S., and De Graaf, A.A., A universal framework for ^{13}C metabolic flux analysis, *Metabolic Engineering*, 3:265–283, 2001.
- [12] Wiechert, W., Siefke, C., De Graaf, A.A., and Marx, A., Bidirectional reaction steps in metabolic networks II: Flux estimation and statistical analysis, *Biotechnology and Bioengineering*, 55(1):118–135, 1997.
- [13] Wittmann, C. and Heinzle, E., Mass spectrometry for metabolic flux analysis, *Biotechnology and Bioengineering*, 62(6):739–751, 1999.
- [14] Zupke, C. and Stephanopoulos, G., Modeling of isotope distributions and intracellular fluxes in metabolic networks using atom mapping matrices, *Biotechnol. Prog.*, 10:489–498, 1994.