Bidirectional Reaction Steps in Metabolic Networks Part III: Explicit Solution and Analysis of Isotopomer Labeling Systems

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Abstract: The last few years have brought tremendous progress in experimental methods for metabolic flux determination by carbon labeling experiments. A significant enlargement of the available measurement data set has been achieved especially when isotopomer fractions within intracellular metabolite pools are quantitated. This information can be used to improve the statistical quality of flux estimates. Furthermore, several assumptions on bidirectional intracellular reaction steps that were hitherto indispensable may now become obsolete. In order to make full use of the complete measurement information a general mathematical model for isotopomer systems is established in this contribution. Then by introducing the important new concept of cumomers and cumomer fractions it is shown that the arising nonlinear isotopomer balance equations can always be analytically solved. In particular the solution of the the metabolite flux balances and the positional carbon labeling balances presented in Part I of this series turn out to be just the first two steps of the general solution procedure for isotopomer balances. A detailed analysis of the isotopomer network structure then opens up new insights into the intrinsic structure of isotopomer systems. In particular it turns out that isotopomer systems are not as complex as they appear at first glance. This enables some far-reaching conclusions to be drawn on the information potential of isotopomer experiments with respect to flux identification. Finally, some illustrative examples are discussed to show that an information increase is not guaranteed when isotopomer measurements are used in addition to positional enrichment data.

Keywords: metabolic flux analysis, ^{13}C isotope labeling experiments, isotopomers, cumomers, network analysis, parameter identifiability

1 Introduction

Parts I and II of this series (Wiechert & de Graaf, 1997; Wiechert *et al.*, 1997a) (henceforth simply called Parts I and II) dealt with modeling, simulation and statistical data analysis for positional carbon labeling experiments. This theoretical development was driven by the necessity to quantitate bidirectional reaction steps in the metabolic network, in order to attain an accurate, comprehensive flux analysis based on ^{13}C labeling experiments. While an optimized analysis was indeed established, it was shown that the evaluation of labeling experiments must always rely on certain assumptions on bidirectional fluxes because the amount of measurement information available from positional ^{13}C labelings is generally not sufficient.

1.1 *Isotopomers*

Isotopomer analysis has the potential to solve this problem. Considering only the ^{12}C and ^{13}C isotopes in the carbon backbone of a molecule M with n carbon atoms an *isotopomer* of M is one of the 2^n possible labeling states in which this molecule can be encountered (Fig. 1) (Malloy *et al.*, 1988). The corresponding *isotopomer fraction* denotes the percentage of molecules in this specific labeling state. The *positional enrichment* at the ith carbon atom M#i within a metabolite M (as treated in Parts I and II) is then the sum of all isotopomer fractions of M where the ith carbon atom is labeled (Fig. 1). An important difference between the two concepts is that the isotopomer fractions of M always sum up to 100 % while positional labeling fractions have no such constraint.

If all isotopomer fractions of a metabolite with n carbon atoms can be measured instead of only positional enrichments an increase from n measured positional enrichments to a maximum of $2^n - 1$ measured isotopomer fractions is achieved (the 2^nth measurement is redundant due to the 100 % constraint). For certain metabolites like sedoheptulose-7-phosphate this will yield a maximal information increase by a factor of $127/7 \approx 18$. Although this factor cannot be reached in practice this illustrates the tremendous potential of isotopomer measurements for flux quantitation compared to positional measurements.

Clearly, by measuring isotopomers one expects an improvement of statistical quality for the flux estimates due to the much higher amount of measured data. Moreover, assumptions about the biochemistry may be dropped. Therefore, the availability of a unifying mathematical modeling framework for both positional labelings and isotopomer distributions would be invaluable.

1.2 *Available measurement data*

Carrying out an isotopomer labeling experiment only makes sense if powerful methods for measuring isotopomer fractions are available and the number and quality of measured values is significantly higher than that of positional labeling data. Fortunately, due to recent experimental progress, these requirements are now met and the corresponding measurement techniques are well developed.

An early application of isotopomer measurements for *in vivo* flux determination is given by (Malloy *et al.*, 1988) where whole animal hearts were studied inside an NMR instrument. This only enabled a single intracellular pool (glutamate) to be observed. Later a series of applications for different systems was reported using NMR (Künnecke *et al.*, 1993; Lapidot & Gopher, 1994) as well as mass spectrometry (MS) (Katz *et al.*, 1993; Donato *et al.*, 1993). In each case only a few measurements were obtained.

This situation has been dramatically changed by recent developments. The most important change is that with the experimental technique of preparing proteinogenic amino acids as introduced in (Marx *et al.*, 1996) the labeling state of many intracellular pools can now be indirectly measured using a retrobiosynthetic approach (Szyperski, 1995). Additionally, since the measurement is performed separately from the actual labeling experiment a high precision can be achieved. This enables 1-dimensional ^{1}H- and ^{13}C-NMR, 2- dimensional ^{1}H-^{13}C-NMR and MS to be applied for isotopomer quantitation yielding a large variety of different measurement data that are directly related to isotopomer fractions. The present situation is reviewed in (Wiechert & de Graaf, 1996; Szyperski, 1998).

More details concerning the different measurement techniques for isotopomers and their modeling will be presented in Part IV of this series. The only basic fact that is important for the understanding of the following is that, in general, the described methods do not enable isotopomer fractions to be directly measured. Instead they all essentially produce linear combinations of such fractions (up to a scaling factor that will be discussed in Part IV). For example, with proton NMR all isotopomers that are labeled on a certain carbon atom position produce the same spectral peak. Likewise an MS measurement peak is (up to a certain isotope effect correction) produced by all isotopomers with the same molecular weight, i.e. the same number of labeled carbon positions. These are also called *mass isotopomers* (Lee *et al.*, 1991). Henceforth the term *isotopomer measurement* is used for any measurable linear combination of isotopomer fractions.

1.3 *Modeling, simulation and data analysis frameworks*

In order to evaluate isotopomer labeling experiments mathematical models are required. The basic principles of isotopomer balancing were first presented in (Jeffrey *et al.*, 1991; Künnecke *et al.*, 1993). Since one balance

equation has to be given for each isotopomer fraction in the system this ends up with about 500 and more equations for the central metabolism. About 65% of the equations are required for glycolysis and the pentose phosphate pathway. For instance 128 equations must be formulated for the sedoheptulose-7-phosphate pool alone! Since previous applications concentrated only on metabolic subsections like the citric acid cycle the number of equations considered has not been that high until now.

Those cases where only part of the isotopomer balances was exploited yielded highly application-specific formulas that cannot be easily generalized to arbitrary networks with less strict assumptions on bidirectionality or differently labeled substrates (Malloy *et al.*, 1988; Lee, 1993; Szyperski, 1995; Klapa *et al.*, 1999). Moreover, such explicit formulas for flux determination do not exploit all the available measurement information, i.e. the statistical quality of the estimated fluxes cannot be improved from redundant data. For this reason all interdependences between fluxes and measurements have to be represented in the model which means that the complete balances must be incorporated in a holistic manner (Schmidt *et al.*, 1997).

Clearly, a manual input of the balance equations is ruled out because this is extremely time- consuming and will almost certainly produce typing errors. For this reason a general mathematical modeling framework accompanied by the appropriate tools for automatic model generation, simulation, parameter estimation and statistical analysis is required, as has been established in Parts I and II for the case of positional labeling systems. For isotopomer systems only parts of such a general framework are currently available (Schmidt *et al.*, 1997).

As was the case with positional carbon labeling, there are basically two formal approaches for establishing a general model structure. The mapping matrix approach recently presented by (Schmidt *et al.*, 1997) generalizes (Zupke & Stephanopoulos, 1994), while the transition matrix approach from (Wiechert, 1996) generalizes (Wiechert & de Graaf, 1997). Of course both approaches are equivalent but use different notations. Mapping matrices describing single reaction steps can be easily used for a quick model implementation using a computer algebra system like MAPLE or a numerical analysis system like MATLAB. On the other hand transition matrices simultaneously describe the whole reaction network and thus are much more suitable for establishing high-performance numerical algorithms and for doing system analysis. In each case the mapping or transition matrices can be automatically generated so that the user is not aware of the technical details of model generation (Schmidt *et al.*, 1997; Möllney *et al.*, 1999).

Given the model equations, several simulation algorithms for general isotopomer labeling systems have been presented. Because isotopomer balance equations are nonlinear, iterative procedures have been used in the past like a modified Euler algorithm (Wiechert, 1996), a modified Jacobi iteration scheme (Schmidt *et al.*, 1997) or a Newton formula (Wiechert *et al.*, 1997b). In each case the presence of large exchange fluxes causes severe instability or convergence problems for each of these algorithms (Wurzel, 1997) which is not surprising because the positional carbon labeling system is known to be ill-conditioned in that case (Wiechert, 1996; Siefke, 1996). Thus more sophisticated algorithms are needed to establish a generally applicable solution.

1.4 *Aims of this contribution*

The aim of this contribution and its sequel is the generalization of all models, methods and tools introduced in Parts I and II to general isotopomer systems. In particular Part III covers the following subjects:

1. The isotopomer balance equations are generally expressed by introducing transition matrices and the software tools for the automatic generation of these complex matrices are supplied.

2. Although the contrary was recently conjectured in (Klapa *et al.*, 1999; Park *et al.*, 1997) it is shown that the nonlinear isotopomer balances can always be analytically solved and an appropriate solution algorithm based on matrix calculus is presented. To this end the important concept of cumomers and cumomer fractions is introduced.

3. The solution algorithm for the isotopomer balances also has a great impact on flux identifiability analysis. For this purpose the concept of cumomer redundancy and the new method of cumomer network analysis is introduced. It represents a powerful tool to gain insight into the information which can be obtained from isotopomer experiments.

4. Some instructive examples will be studied using the newly developed tools. It appears that isotopomer networks are not as complex as suggested by their large dimensionality. Some far-reaching conclusions can be drawn on the identifiability of fluxes and the improvement to be achieved by using isotopomer data.

The statistical analysis of isotopomer experiments and their comparison with positional labeling experiments is carried out in Part IV. This will enable the different methods that are currently being promoted to be compared on the basis of quantitative criteria.

2 Isotopomer Labeling Balances

The principles of formulating isotopomer labeling balances are now briefly presented by using a simple example. This example will then be used throughout the following chapters to introduce the concept of cumomer fractions and to relate them to the isotopomer fractions.

2.1 *A simple example*

The example network with its metabolite fluxes and carbon atom transitions is given in Figure 2. It is modeled on the citric acid cycle together with the anaplerotic reaction section but is simplified to a few metabolites with a maximum of 4 carbon atoms. Using the formal notation for carbon atom transitions introduced in Part I the network has the following structure:

v1:	A			>	B			v5:	B	>	K				
	#xy			>	#xy				#xy	>	#xy				
v2:	B			>	E			v6:	C	>	D	+	F		
	#xy			>	#xy				#uvxy	>	#vxy	+	#u		
v3:	B	+	E	>	C			v7:	D	>	E	+	G		
	#xy	+	#uv	>	#xyuv				#uxy	>	#ux	+	#y		
v4:	E			>	H										
	#xy			>	#xy										

Flux v1 is an input flux and thus assumed unidirectional (i.e. $v_1^{\leftarrow} = 0$). The reason for this directionality convention is that a backflux in v1 would have no effect on the intracellular labelling state (cf. Part I). The fluxes v4, v5, v6, v7 are output fluxes. If one of these fluxes would have a backflux another labeling source from the cell surrounding would have to be introduced into the network. By convention (cf. Part I) such an input is only allowed as a dedicated system influx. Because such an additional input is not assumed in the example the output fluxes are also unidirectional (i.e. $v_4^{\leftarrow} = v_5^{\leftarrow} = v_6^{\leftarrow} = v_7^{\leftarrow} = 0$). The remaining intracellular fluxes v2, v3 are assumed to take place in both directions. This yields the flux balances:

$$\begin{array}{lrcl} \mathsf{B}: & v_1^{\rightarrow} + v_2^{\leftarrow} + v_3^{\leftarrow} & = & v_2^{\rightarrow} + v_3^{\rightarrow} + v_5^{\rightarrow} \\ \mathsf{C}: & v_3^{\rightarrow} & = & v_3^{\leftarrow} + v_6^{\rightarrow} \\ \mathsf{D}: & v_6^{\rightarrow} & = & v_7^{\rightarrow} \\ \mathsf{E}: & v_2^{\rightarrow} + v_3^{\leftarrow} + v_7^{\rightarrow} & = & v_2^{\leftarrow} + v_3^{\rightarrow} + v_4^{\rightarrow} \end{array} \tag{1}$$

Choosing $v_1^{\rightarrow}$, $v_2^{\rightarrow}$, $v_2^{\leftarrow}$, $v_3^{\rightarrow}$, $v_3^{\leftarrow}$ as the free fluxes the remaining fluxes are expressed as:

$$\begin{array}{lcl} v_6^{\rightarrow} = v_7^{\rightarrow} & = & v_3^{\rightarrow} - v_3^{\leftarrow} \\ v_4^{\rightarrow} & = & v_2^{\rightarrow} - v_2^{\leftarrow} \\ v_5^{\rightarrow} & = & v_1^{\rightarrow} + v_2^{\leftarrow} - v_2^{\rightarrow} + v_3^{\leftarrow} - v_3^{\rightarrow} \end{array} \tag{2}$$

2.2 *Isotopomer fractions*

The isotopomers of a metabolite M are denoted by using an obvious binary notation as M#abc... with a,b,c,...= 0 or 1. Here a 1 indicates that the corresponding carbon atom position is labeled and a 0 indicates that it is not labeled. For example C#0101 denotes the isotopomer of C which is labeled at the second and fourth position.

The state variables usually used for the description of the system's isotopomer labeling state are the isotopomer fractions of all input and intracellular metabolites (see Fig. 1). For the input metabolite A this yields $2^2 = 4$ variables and for the intracellular metabolites B, C, D, E this yields $2^2+2^4+2^3+2^2 = 32$ variables. The isotopomer fractions of M are denoted by using an index notation corresponding to the isotopomer name as $m_{abc...}$. For example the isotopomer fractions of D are written as $d_{000}, d_{001}, d_{010}, d_{011}, d_{100}, d_{101}, d_{110}, d_{111}$. In the following the isotopomer fractions will also be denoted with a more compact notation by using indices $i, j, k, l \in \{0, 1\}$ as:

$$a_{ij},\ b_{ij},\ c_{ijkl},\ d_{ijk},\ e_{ij}, \qquad i, j, k, l = 0, 1$$

Clearly, the sum of all isotopomer fractions corresponding to one metabolite is 100%, i.e.

$$\sum_{i,j=0}^{1} a_{ij} = 1,\ \sum_{i,j=0}^{1} b_{ij} = 1,\ \sum_{i,j,k,l=0}^{1} c_{ijkl} = 1,\ \sum_{i,j,k=0}^{1} d_{ijk} = 1,\ \sum_{i,j=0}^{1} e_{ij} = 1 \quad . \tag{3}$$

2.3 *Balance equations*

Assuming isotopic stationarity (Marx *et al.*, 1996; Wiechert, 1996) a balance equation can now be formulated for each of the 32 intracellular isotopomer pools as has been already described in (Wiechert & de Graaf, 1996; Schmidt *et al.*, 1997). As opposed to positional labeling systems, unimolecular and bimolecular reaction steps must be treated separately. Furthermore a distinction must be made between bimolecularity on the educt side and on the product side.

As an example of bimolecularity on the educt side the balance for the pool C#1001 is given by:

$$\mathsf{C\#1001}: c_{1001}\,(v_3^{\leftarrow} + v_6^{\rightarrow}) = b_{10}\,e_{01}\,v_3^{\rightarrow} \tag{4}$$

Here the effluxes are collected on the left side and the influxes are collected on the right side. The efflux is given by the total amount of molecules carried out of the metabolite pool C by the fluxes $v_3^{\leftarrow}$ and $v_6^{\rightarrow}$ times the percentage of the isotopomer considered (i.e. c_{1001}). On the right-hand side a product of the isotopomer fractions b_{10} and e_{01} occurs because the target isotopomer is formed from two educt isotopomers. The product is the probability that both educt isotopomers happen to be combined by the bimolecular reaction step v3.

The right side of Equation (4) is nonlinear with respect to the isotopomer fractions because the quadratic term $b_{10}\,e_{01}$ occurs. This means that isotopomer balance equations cannot be simply written by using transition matrices as in Part I and that they cannot be easily solved for the labeling variables when all fluxes are known. However, such a quadratic term only occurs when a metabolite is formed in a bimolecular reaction step and in all other cases the arising terms are linear as is shown below.

Another important difference between positional and isotopomer balances occurs when there is a bimolecularity on the product side. When an educt metabolite is split into parts the product isotopomers (unlike carbon atoms) can be obtained from more than one educt isotopomer. This is shown by the example:

$$\mathsf{B\#10}: \; b_{10}\,(v_2^{\rightarrow} + v_3^{\rightarrow} + v_5^{\rightarrow}) = (c_{1000} + c_{1001} + c_{1010} + c_{1011})\,v_3^{\leftarrow} + a_{10}\,v_1^{\rightarrow} + e_{10}\,v_2^{\leftarrow} \tag{5}$$

Here all educt isotopomers C#1000, C#1001, C#1010,C#1011 yield the same product B#10 in reaction step v3.

A reaction step that is bimolecular on the educt and on the product side like e.g.

w: M + N > P + Q

can be easily reduced to the two cases discussed above by introducing an intermediate metabolite and splitting the reaction into two steps:

w1: M + N > MN w2: MN > P + Q

Finally, the balances for unidirectional reaction steps are obtained by the same principles as for positional labeling systems. All balance equations are finally summarized by using indices $i, j, k, l \in \{0, 1\}$ in the compact notation:

$$\begin{array}{lrcl}
\mathsf{B\#ij}: & b_{ij}\quad (v_2^{\rightarrow} + v_3^{\rightarrow} + v_5^{\rightarrow}) & = & (c_{ij00} + c_{ij01} + c_{ij10} + c_{ij11})\,v_3^{\leftarrow} + a_{ij}\,v_1^{\rightarrow} + e_{ij}\,v_2^{\leftarrow} \\
\mathsf{C\#ijkl}: & c_{ijkl}\,(v_3^{\leftarrow} + v_6^{\rightarrow}) & = & b_{ij}\,e_{kl}\,v_3^{\rightarrow} \\
\mathsf{D\#ijk}: & d_{ijk}\quad v_7^{\rightarrow} & = & (c_{0ijk} + c_{1ijk})\,v_6^{\rightarrow} \\
\mathsf{E\#ij}: & e_{ij}\quad (v_2^{\leftarrow} + v_3^{\rightarrow} + v_4^{\rightarrow}) & = & (c_{00ij} + c_{01ij} + c_{10ij} + c_{11ij})\,v_3^{\leftarrow} + (d_{ij0} + d_{ij1})\,v_7^{\rightarrow} + b_{ij}\,v_2^{\rightarrow}
\end{array} \tag{6}$$

These 32 equations must be combined with Equation (3) so that there are finally more equations than fractional variables. This is explained by a redundancy in the combined equation set (6) that is obtained by adding up all balance equations corresponding to one metabolite. For instance all balances for the pool B add up to:

$$\overbrace{\left(\sum_{i,j=0}^{1} b_{ij}\right)}^{=1}(v_2^{\rightarrow} + v_3^{\rightarrow} + v_5^{\rightarrow}) \quad = \quad \underbrace{\left(\sum_{i,j=0}^{1} c_{00ij} + c_{01ij} + c_{10ij} + c_{11ij}\right)}_{=1} v_3^{\leftarrow} + \underbrace{\left(\sum_{i,j=0}^{1} a_{ij}\right)}_{=1} v_1^{\rightarrow} + \underbrace{\left(\sum_{i,j=0}^{1} e_{ij}\right)}_{=1} v_2^{\leftarrow}$$

This is exactly the metabolite flux balance for pool B from Equation (1).

3 Cumomer Labeling Balances

At first glance there is no way to solve the isotopomer balance equations analytically due to their nonlinear structure and high dimensionality. This has given rise to the different iterative numerical solution approaches mentioned in the Introduction. Surprisingly, after a suitable variable transformation the equations can be always explicitly solved. After transforming the equations they have a much simpler but still familiar structure.

3.1 *Cumomer fractions*

The transformed variables will be called *cumomer fractions*. The artificial word "cumomer fraction" is an abbreviation for "cumulated isotopomer fraction" and means a certain sum of isotopomer fractions of a metabolite. Cumomer fractions are introduced by the running example for metabolite D. The so-called 0-cumomer fraction of D is simply the sum of all its isotopomer fractions, i.e.

$$d_{xxx} \stackrel{def}{=} \sum_{i,j,k=0}^{1} d_{ijk} = 1 \quad . \tag{7}$$

Here the index x has the obvious meaning "0 or 1". Using the same notational convention the 1-cumomer fractions of D are obtained as:

$$d_{1xx} \stackrel{def}{=} \sum_{j,k=0}^{1} d_{1jk}, \quad d_{x1x} \stackrel{def}{=} \sum_{i,k=0}^{1} d_{i1k}, \quad d_{xx1} \stackrel{def}{=} \sum_{i,j=0}^{1} d_{ij1} \tag{8}$$

Thus the 1-cumomer fractions are the percentages of all isotopomers that are labeled at least at the single position indicated by the index 1. Of course, these are exactly the familiar positional labeling fractions $d_1 = d_{1xx},\ d_2 = d_{x1x},\ d_3 = d_{xx1}$ introduced in Part I.

Continuing the idea of cumulative isotopomer fractions the 2-cumomer fractions are formed from all isotopomers with at least two specified labeled carbon atoms as indicated by the index 1:

$$d_{11x} \stackrel{def}{=} \sum_{k=0}^{1} d_{11k}, \quad d_{1x1} \stackrel{def}{=} \sum_{j=0}^{1} d_{1j1}, \quad d_{x11} \stackrel{def}{=} \sum_{i=0}^{1} d_{i11} \tag{9}$$

Finally, there is the single 3-cumomer fraction d_{111} which is identical to the corresponding isotopomer fraction. It is shown in Section "The General Model" that the linear transformation

$$(d_{000}, d_{001}, d_{010}, d_{011}, d_{100}, d_{101}, d_{110}, d_{111}) \longleftrightarrow (d_{xxx}, d_{xx1}, d_{x1x}, d_{x11}, d_{1xx}, d_{1x1}, d_{11x}, d_{111})$$

is always a one-to-one correspondence, i.e. the cumomer fractions can be calculated from the isotopomer fractions and vice versa.

From now on the term *cumomer* is used to denote a "virtual molecule", to which a cumomer fraction is assigned. For example, the notation C#1xx1 is used for a cumomer and c_{1xx1} for the corresponding cumomer fraction. Clearly, a cumomer is not a real particle but rather a set of different isotopomers. However, this terminology makes it more convenient to talk about the cumomer balance equations introduced in the next section. In particular a *cumomer network* can be constructed that is in the same relation to the cumomer balances as the isotopomer network is to the isotopomer balances.

3.2 *Balance equations*

The cumomer balances are computed by transformation of the isotopomer balances. This is achieved by summing up the equations of all isotopomers belonging to a certain cumomer. As an example, to obtain the cumomer balance equation for the cumomer C#1xx1 the equations for the isotopomers C#1001, C#1011, C#1101 and C#1111 have to be summed up. The result is:

$$\text{C\#1xx1}: \underbrace{\left(\sum_{i,j=0}^{1} c_{1ij1}\right)}_{=c_{1xx1}} (v_3^{\leftarrow} + v_6^{\rightarrow}) = \sum_{i,j=0}^{1} b_{1i}\, e_{j1}\, v_3^{\rightarrow} = \underbrace{\left(\sum_{i=0}^{1} b_{1i}\right)}_{=b_{1x}} \underbrace{\left(\sum_{j=0}^{1} e_{j1}\right)}_{=e_{x1}} v_3^{\rightarrow} \tag{10}$$

This shows that in the case of a bimolecular product the corresponding cumomer balances can be simply constructed from the isotopomer balances by replacing each index 0 in Equation (4) by x. This is not so simple for the product of a splitting reaction step as can be observed for B#1x:

$$\text{B\#1x}: \underbrace{\left(\sum_{i=0}^{1} b_{1i}\right)}_{=b_{1x}} (v_2^{\rightarrow} + v_3^{\rightarrow} + v_5^{\rightarrow}) \quad =$$

$$\underbrace{\left(\sum_{i=0}^{1} c_{1i00} + c_{1i01} + c_{1i10} + c_{1i11}\right)}_{=c_{1xxx}} v_3^{\leftarrow} + \underbrace{\left(\sum_{i=0}^{1} a_{1i}\right)}_{=a_{1x}} v_1^{\rightarrow} + \underbrace{\left(\sum_{i=0}^{1} e_{1i}\right)}_{=e_{1x}} v_2^{\leftarrow} \tag{11}$$

This is simply the carbon balance equation for B#1 written in an unfamiliar notation. But more important is the fact that the original sum $c_{1000} + c_{1001} + c_{1010} + c_{1011}$ from Equation (5) is reduced to only one cumomer term c_{1xxx}, i.e. the index replacement rule "$0 \rightarrow x$" does not hold in this situation.

3.3 *Weight preservation*

To understand the general principle of cumomer balance formulation the key concept of the *weight* of each isotopomer or cumomer is defined. The weight of an isotopomer denotes the number of its labeled carbon atoms, i.e. for example

$$\text{weight (B\#ij)} = i + j, \quad \text{weight (C\#ijkl)} = i + j + k + l, \quad \text{weight (D\#ijk)} = i + j + k \quad .$$

Likewise the weight of an n-cumomer is defined to be n, i.e. the weight of the isotopomer that is created by replacing the letter X in the cumomer notation with 0. For example

$$\text{weight (C\#1XX1)} = \text{weight (C\#1001)} = 2 \quad .$$

The term "weight" is also used for the corresponding labeling variables, i.e. $b_{ij}, c_{ijkl}, d_{ijk}$.

The general rule now becomes clear by observing that cumomer balances are always *weight preserving*. This means that in the balance Equation (11) for the 1-cumomer B#1x all the involved labeling fractions $a_{1x}, b_{1x}, c_{1xxx}, e_{1x}$ correspond to cumomers with weight 1. The same rule applies for the 2-cumomer fraction C#1xx1 from Equation (10), if the additional convention is made that the weight of a quadratic term is the sum of its factor weights. Thus the quadratic term $b_{1x}\, e_{x1}$ and the linear term c_{1xx1} both have weight 2 in Equation (10).

Weight preservation does not hold for the isotopomer balances. For example, the isotopomer fractions $c_{1000}, c_{1001}, c_{1010}, c_{1011}$ of weight $1, 2, 3$ are all involved in the balance for B#10 from Equation (5). So the general procedure for converting isotopomer into cumomer balances is as follows:

$$\begin{array}{ll} 1. & \text{First replace each index } 0 \text{ by } x \text{ in all isotopomer balance equations.} \\ 2. & \text{Then remove all sum terms that are not weight-preserving.} \end{array} \qquad (12)$$

The correctness of these rules can be generally proven for arbitrary networks (Wurzel, 1997). The complete cumomer balances for the running example can thus be compactly written by using indices $i, j, k, l \in \{x, 1\}$ as:

$$\begin{array}{lrcl} \text{B\#ij} & b_{ij}\ (v_2^{\rightarrow} + v_3^{\rightarrow} + v_5^{\rightarrow}) & = & c_{ijxx}\, v_3^{\leftarrow} + a_{ij}\, v_1^{\rightarrow} + e_{ij}\, v_2^{\leftarrow} \\ \text{C\#ijkl} & c_{ijkl}\ (v_3^{\leftarrow} + v_6^{\rightarrow}) & = & b_{ij}\, e_{kl}\, v_3^{\rightarrow} \\ \text{D\#ijk} & d_{ijk}\ v_7^{\rightarrow} & = & c_{xijk}\, v_6^{\rightarrow} \\ \text{E\#ij} & e_{ij}\ (v_2^{\leftarrow} + v_3^{\rightarrow} + v_4^{\rightarrow}) & = & c_{xxij}\, v_3^{\leftarrow} + d_{ijx}\, v_7^{\rightarrow} + b_{ij}\, v_2^{\rightarrow} \end{array} \qquad (13)$$

The reader should verify these equations by comparison with Equation (6).

3.4 *Solution of the example system*

The cumomer labeling balances from Equation (13) turn out to be slightly simpler than the isotopomer balances from Equation (6) because non-weight-preserving terms are omitted. This has dramatic consequences for the solution of the equations because the cumomer balance equation for an n-cumomer can only contain cumomer fractions with a weight less then or equal to n. Consequently, the cumomer balances are less strongly coupled than the isotopomer balances.

The second important observation is that in an n-cumomer balance a fraction variable of weight less than n can only occur as a factor of a bilinear term. In particular the factors of this term have either both weights less than n or one weight is n and the other is 0. Since a 0-cumomer fraction has value 1 by definition the 0-cumomer fractions can be left out so that only the n-cumomer factor remains. Consequently, the terms of weight n always occur linearly in an n-cumomer balance equation. This is the key for solving them explicitly.

To demonstrate this by the running example a cascade of linear equations is constructed from which the 1-, 2-, 3-...cumomer fractions are successively computed. We start with the 0-cumomers. The corresponding equations are from Equation (13):

$$\begin{array}{lrcccc} \text{B\#xx} & b_{xx}\ (v_2^{\rightarrow} + v_3^{\rightarrow} + v_5^{\rightarrow}) & -c_{xxxx}\ v_3^{\leftarrow} & -e_{xx}\ v_2^{\leftarrow} & & = a_{xx}\, v_1^{\rightarrow} \\ \text{C\#xxxx} & c_{xxxx}\ (v_3^{\leftarrow} + v_6^{\rightarrow}) & -b_{xx}\, e_{xx}\, v_3^{\rightarrow} & & & = 0 \\ \text{D\#xxx} & d_{xxx}\ v_7^{\rightarrow} & -c_{xxxx}\ v_6^{\rightarrow} & & & = 0 \\ \text{E\#xx} & e_{xx}\ (v_2^{\leftarrow} + v_3^{\rightarrow} + v_4^{\rightarrow}) & -c_{xxxx}\ v_3^{\leftarrow} & -d_{xxx}\, v_7^{\rightarrow} & -b_{xx}\, v_2^{\rightarrow} & = 0 \end{array} \qquad (14)$$

Since all 0-cumomer fractions are 1 these are exactly the metabolite flux balances from Equation (1).

It is now continued with the 1-cumomer fractions which are exactly the positional carbon labeling equations from Part I. Here all 1-cumomer terms have been arranged on the left side and the 0-cumomer fractions have been

eliminated. The known input cumomer fractions a_{1x}, a_{x1} can be found on the right side:

$$
\begin{array}{lll}
\mathsf{B\#x1} & : b_{x1} \quad (v_2^{\rightarrow} + v_3^{\rightarrow} + v_5^{\rightarrow}) & -c_{x1xx}\, v_3^{\leftarrow} \quad -e_{x1} \quad v_2^{\leftarrow} \quad = a_{x1}\, v_1^{\rightarrow} \\
\mathsf{B\#1x} & : b_{1x} \quad (v_2^{\rightarrow} + v_3^{\rightarrow} + v_5^{\rightarrow}) & -c_{1xxx}\, v_3^{\leftarrow} \quad -e_{1x} \quad v_2^{\leftarrow} \quad = a_{1x}\, v_1^{\rightarrow} \\
\\
\mathsf{C\#1xxx}: & c_{1xxx}\,(v_3^{\leftarrow} + v_6^{\rightarrow}) & -b_{1x} \quad v_3^{\rightarrow} \quad = 0 \\
\mathsf{C\#x1xx}: & c_{x1xx}\,(v_3^{\leftarrow} + v_6^{\rightarrow}) & -b_{x1} \quad v_3^{\rightarrow} \quad = 0 \\
\mathsf{C\#xx1x}: & c_{xx1x}\,(v_3^{\leftarrow} + v_6^{\rightarrow}) & -e_{1x} \quad v_3^{\rightarrow} \quad = 0 \\
\mathsf{C\#xxx1}: & c_{xxx1}\,(v_3^{\leftarrow} + v_6^{\rightarrow}) & -e_{x1} \quad v_3^{\rightarrow} \quad = 0 \\
\\
\mathsf{D\#1xx} & : d_{1xx} \quad v_7^{\rightarrow} & -c_{x1xx}\, v_6^{\rightarrow} \quad = 0 \\
\mathsf{D\#x1x} & : d_{x1x} \quad v_7^{\rightarrow} & -c_{xx1x}\, v_6^{\rightarrow} \quad = 0 \\
\mathsf{D\#xx1} & : d_{xx1} \quad v_7^{\rightarrow} & -c_{xxx1}\, v_6^{\rightarrow} \quad = 0 \\
\\
\mathsf{E\#1x} & : e_{1x} \quad (v_2^{\leftarrow} + v_3^{\rightarrow} + v_4^{\rightarrow}) & -c_{xx1x}\, v_3^{\leftarrow} \quad -d_{1xx}\, v_7^{\rightarrow} \quad -b_{1x}\, v_2^{\rightarrow} \quad = 0 \\
\mathsf{E\#x1} & : e_{x1} \quad (v_2^{\leftarrow} + v_3^{\rightarrow} + v_4^{\rightarrow}) & -c_{xxx1}\, v_3^{\leftarrow} \quad -d_{x1x}\, v_7^{\rightarrow} \quad -b_{x1}\, v_2^{\rightarrow} \quad = 0
\end{array}
\tag{15}
$$

From this linear equation system the 1-cumomer fractions can be computed as a function of the free fluxes with the help of a computer algebra system. The resulting lengthy formulas are not reproduced here for the sake of brevity.

Going over to the 2-cumomer fractions, all 1-cumomer fractions can be assumed to be known and thus are put on the right side:

$$
\begin{array}{lll}
\mathsf{B\#11} & : b_{11} \quad (v_2^{\rightarrow} + v_3^{\rightarrow} + v_5^{\rightarrow}) & -c_{11xx}\, v_3^{\leftarrow} \quad -e_{11} \quad v_2^{\leftarrow} \quad = a_{11} \quad v_1^{\rightarrow} \\
\\
\mathsf{C\#11xx}: & c_{11xx}\,(v_3^{\leftarrow} + v_6^{\rightarrow}) & -b_{11} \quad v_3^{\rightarrow} \quad = 0 \\
\mathsf{C\#1x1x}: & c_{1x1x}\,(v_3^{\leftarrow} + v_6^{\rightarrow}) & = b_{1x}\, e_{1x}\, v_3^{\rightarrow} \\
\mathsf{C\#1xx1}: & c_{1xx1}\,(v_3^{\leftarrow} + v_6^{\rightarrow}) & = b_{1x}\, e_{x1}\, v_3^{\rightarrow} \\
\mathsf{C\#x11x}: & c_{x11x}\,(v_3^{\leftarrow} + v_6^{\rightarrow}) & = b_{x1}\, e_{1x}\, v_3^{\rightarrow} \\
\mathsf{C\#x1x1}: & c_{x1x1}\,(v_3^{\leftarrow} + v_6^{\rightarrow}) & = b_{x1}\, e_{x1}\, v_3^{\rightarrow} \\
\mathsf{C\#xx11}: & c_{xx11}\,(v_3^{\leftarrow} + v_6^{\rightarrow}) & -e_{11} \quad v_3^{\rightarrow} \quad = 0 \\
\\
\mathsf{D\#11x} & : d_{11x} \quad v_7^{\rightarrow} & -c_{x11x}\, v_6^{\rightarrow} \quad = 0 \\
\mathsf{D\#1x1} & : d_{1x1} \quad v_7^{\rightarrow} & -c_{x1x1}\, v_6^{\rightarrow} \quad = 0 \\
\mathsf{D\#x11} & : d_{x11} \quad v_7^{\rightarrow} & -c_{xx11}\, v_6^{\rightarrow} \quad = 0 \\
\\
\mathsf{E\#11} & : e_{11} \quad (v_2^{\leftarrow} + v_3^{\rightarrow} + v_4^{\rightarrow}) & -c_{xx11}\, v_3^{\leftarrow} \quad -d_{11x}\, v_7^{\rightarrow} \quad -b_{11}\, v_2^{\rightarrow} \quad = 0
\end{array}
\tag{16}
$$

Again the solution of this linear equation system is not given here for shortness.

There are only a few 3-cumomer equations because they can only occur in C and D:

$$
\begin{array}{lll}
\mathsf{C\#111x}: & c_{111x}\,(v_3^{\leftarrow} + v_6^{\rightarrow}) & = b_{11}\, e_{1x}\, v_3^{\rightarrow} \\
\mathsf{C\#11x1}: & c_{11x1}\,(v_3^{\leftarrow} + v_6^{\rightarrow}) & = b_{11}\, e_{x1}\, v_3^{\rightarrow} \\
\mathsf{C\#1x11}: & c_{1x11}\,(v_3^{\leftarrow} + v_6^{\rightarrow}) & = b_{1x}\, e_{11}\, v_3^{\rightarrow} \\
\mathsf{C\#x111}: & c_{x111}\,(v_3^{\leftarrow} + v_6^{\rightarrow}) & = b_{x1}\, e_{11}\, v_3^{\rightarrow} \\
\\
\mathsf{D\#111} & : d_{111} \quad v_7^{\rightarrow} & -c_{x111}\, v_6^{\rightarrow} \quad = 0
\end{array}
\tag{17}
$$

Finally, the only 4-cumomer fraction in the system is described by:

$$
\mathsf{C\#1111}: \quad c_{1111}\,(v_3^{\leftarrow} + v_6^{\rightarrow}) \quad = \quad b_{11}\, e_{11}\, v_3^{\rightarrow} \tag{18}
$$

By successive substitution of the analytically computed $0, 1, \ldots, n-1$-cumomer fractions a representation of the n-cumomer fractions is obtained in terms of the known input cumomer fractions a_{ij} and the free fluxes. From this the isotopomer fractions are computed by using the linear transformations from Equations (7-9). Thus as a main result the cumomer and isotopomer fractions are always rational functions of the input fractions and the free fluxes. Moreover, the cumomer balance equation system presents a unifying formalism for metabolite flux balancing (0-cumomer balances), positional carbon fraction balancing (1-cumomer balances) and isotopomer fraction balancing.

4 The General Model

All procedures that have been demonstrated by the example are now carried over to a more abstract matrix notation that is suitable for computer implementation, numerical computations and systems analysis. We restrict ourselves to networks containing only unimolecular and bimolecular reaction steps. Apart from the fact that the central metabolism does not contain any reaction step with three or more labeled partners on the educt side, this situation can be easily handled by replacing a reaction step

$$
\mathsf{A + B + C > D + E + F}
$$

by the sequence

$$\mathsf{A + B + C > AB + C,} \qquad \mathsf{AB + C > DE + F,} \qquad \mathsf{DE + F > D + E + F} \quad .$$

The restriction to bimolecular steps will keep the formal efforts low in the following.

4.1 *Isotopomer and cumomer state vectors*

As has been done with the positional labeling fractions, all input isotopomer fractions and all intermediate isotopomer fractions are numbered consecutively and collected within the vectors $\overline{\mathbf{x}}^{\text{inp}}$ and $\overline{\mathbf{x}}$. In the example they are given by

$$\begin{aligned} \overline{\mathbf{x}}^{\text{inp}} &= (a_{00}, a_{01}, a_{10}, a_{11})^T \qquad (19) \\ \text{and} \quad \overline{\mathbf{x}} &= (b_{00}, b_{01}, b_{10}, b_{11}, \\ &\quad c_{0000}, c_{0001}, c_{0010}, c_{0011}, c_{0100}, c_{0101}, c_{0110}, c_{0111}, \\ &\quad\; c_{1000}, c_{1001}, c_{1010}, c_{1011}, c_{1100}, c_{1101}, c_{1110}, c_{1111}, \\ &\quad d_{000}, d_{001}, d_{010}, d_{011}, d_{100}, d_{101}, d_{110}, d_{111}, \\ &\quad e_{00}, e_{01}, e_{10}, e_{11} \qquad)^T \quad . \end{aligned}$$

Here the variables are first arranged by the metabolites they belong to. Secondly, within each metabolite they are arranged by their index that is interpreted as a binary number. This type of ordering is henceforth called a *binary ordering* as opposed to a *weight ordering* that will be used in the appendix.

Similar to the isotopomer fractions, the cumomer fractions are collected within the vectors $\mathbf{x}^{\text{inp}}$ and $\mathbf{x}$ which by convention are always ordered in the same way as the isotopomer fractions (i.e. binary or by weight). In the following, the bar decoration always indicates that this vector or matrix belongs to the isotopomers while nondecorated vectors or matrices belong to the cumomers.

4.2 *3-dimensional matrices*

In order to formulate the isotopomer and cumomer balance equations with a formalism that is similar to that introduced for positional labeling in Part I it is necessary to introduce a matrix notation that helps to express the newly arising quadratic terms. Usually quadratic terms in the state variables $\mathbf{x}$ are written with a symmetric square matrix $\mathbf{M}$ as $\mathbf{x}^T\,\mathbf{M}\,\mathbf{x}$. For example the cumomer balance from Equation (10) can be formulated as

$$\mathsf{C\#1xx1} : 0 = \frac{1}{2}\,\mathbf{x}^T \cdot \underbrace{\begin{pmatrix} \ddots & \vdots & \ddots & \vdots & \ddots \\ \cdots & \cdot & \cdots & 1 & \cdots \\ \ddots & \vdots & \ddots & \vdots & \ddots \\ \cdots & 1 & \ddots & \vdots & \vdots \\ \ddots & \vdots & \ddots & \vdots & \ddots \end{pmatrix}}_{\mathbf{Q}^{\rightarrow}_{3,1xx1}} \overset{b_{1x}\ \downarrow \qquad e_{x1}\ \downarrow}{\cdot \mathbf{x} \cdot v_3^{\rightarrow} + (\ \cdots \quad -1 \quad \cdots\ ) \overset{c_{1xx1}\ \downarrow}{\cdot} \mathbf{x} \cdot (v_3^{\leftarrow} + v_6^{\rightarrow})}$$

where the dots indicate zero entries. The factor $1/2$ ensures that the quadratic term $b_{1x}\, e_{x1}$ is not counted twice in the matrix $\mathbf{Q}^{\rightarrow}_{3,1xx1}$. The symmetry of $\mathbf{Q}^{\rightarrow}_{3,1xx1}$ will be a useful property later on (see Appendix A).

One such square matrix $\mathbf{Q}^{\rightarrow}_{i,j}$ or $\mathbf{Q}^{\leftarrow}_{i,j}$ has to be constructed for each bimolecular flux $v_i^{\rightarrow}$ or $v_i^{\leftarrow}$ and for each target cumomer fraction $\mathbf{x}_j$. Herein a nonzero entry $\left(\mathbf{Q}^{\rightarrow}_{i,j}\right)_{k,l}$ corresponds to two cumomers with index k and l that contribute to the balance of $\mathbf{x}_j$ through a bimolecular reaction step $\mathbf{v}_i^{\rightarrow}$.

In order to obtain a more compact notation the matrices $\mathbf{Q}^{\rightarrow}_{i,j}$, $j = 1, 2, \ldots$ are now combined to a 3-dimensional matrix

$$\mathbf{Q}_i^{\rightarrow} = \begin{pmatrix} \mathbf{Q}^{\rightarrow}_{i,1} \\ \vdots \\ \mathbf{Q}^{\rightarrow}_{i,\dim \mathbf{x}} \end{pmatrix}$$

and $\mathbf{Q}_i^{\leftarrow}$ is formed analogously. Then a vector-valued vector-matrix-vector product is defined to be

$$\mathbf{x}^T\,\mathbf{Q}_i^{\rightarrow}\,\mathbf{x} = \begin{pmatrix} \mathbf{x}^T\,\mathbf{Q}^{\rightarrow}_{i,1}\,\mathbf{x} \\ \vdots \\ \mathbf{x}^T\,\mathbf{Q}^{\rightarrow}_{i,\dim \mathbf{x}}\,\mathbf{x} \end{pmatrix}$$

and similarly for $\mathbf{Q}_i^{\leftarrow}$. The vector-valued term $\mathbf{x}^T\,\mathbf{Q}_i^{\rightarrow}\,\mathbf{x}$ can now be used together with the matrix-vector products for unimolecular transitions from Part I, (i.e. $\mathbf{P}_i^{\rightarrow}\,\mathbf{x}, \mathbf{P}_i^{\leftarrow}\,\mathbf{x}$ and $\mathbf{P}_i^{\text{inp}}\,\mathbf{x}$) to formulate the cumomer balances. In the same way the matrices $\overline{\mathbf{P}}_i^{\rightarrow}, \overline{\mathbf{P}}_i^{\leftarrow}, \overline{\mathbf{P}}_i^{\text{inp}}$ and the 3-dimensional matrices $\overline{\mathbf{Q}}_i^{\rightarrow}, \overline{\mathbf{Q}}_i^{\leftarrow}$ will be used to express the isotopomer balance equations.

4.3 *General matrix notation of the balance equations*

Before the cumomer balance equations can be formally written, the isotopomer balance equations have to be specified first. Using the notation for quadratic terms introduced above and keeping in mind that isotopomer related terms are written with a bar decoration the general isotopomer labeling balances can be formulated in a compact way as

$$\frac{1}{2}\,\overline{\mathbf{x}}^T \cdot \left(\sum_i \mathbf{v}_i^{\rightarrow} \cdot \overline{\mathbf{Q}}_i^{\rightarrow} + \mathbf{v}_i^{\leftarrow} \cdot \overline{\mathbf{Q}}_i^{\leftarrow}\right) \cdot \overline{\mathbf{x}} + \left(\sum_i \mathbf{v}_i^{\rightarrow} \cdot \overline{\mathbf{P}}_i^{\rightarrow} + \mathbf{v}_i^{\leftarrow} \cdot \overline{\mathbf{P}}_i^{\leftarrow}\right) \cdot \overline{\mathbf{x}} + \left(\sum_i \mathbf{v}_i^{\rightarrow} \cdot \overline{\mathbf{P}}_i^{\text{inp}}\right) \cdot \overline{\mathbf{x}}^{\text{inp}} = \mathbf{0} \qquad (20)$$

with the bimolecular isotopomer transition matrices $\overline{\mathbf{Q}}_i^{\rightarrow}, \overline{\mathbf{Q}}_i^{\leftarrow}$, the unimolecular isotopomer transition matrices $\overline{\mathbf{P}}_i^{\rightarrow}, \overline{\mathbf{P}}_i^{\leftarrow}$ and the unimolecular input isotopomer transition matrices $\overline{\mathbf{P}}_i^{\text{inp}}$. It should be noted that bilinear terms are not required for input metabolites because the latter must enter into the system by a unimolecular step through the convention made in Part I. The precise definition of the bimolecular transition matrices is given as follows:

$$(\overline{\mathbf{Q}}_{i,j}^{\rightarrow})_{k,l} = \begin{cases} 1 & \text{if the } i\text{th forward reaction step combines the isotopomers} \\ & \text{with index } k \text{ and } l \text{ to the isotopomer with index } j \\ 0 & \text{else} \end{cases} \qquad (21)$$

It follows immediately that $\overline{\mathbf{Q}}_{i,j}^{\rightarrow}$ is a symmetric matrix. The unimolecular transition matrices are defined in the same way as for positional carbon fractions:

$$(\overline{\mathbf{P}}_i^{\rightarrow})_{j,k} = \begin{cases} 1 & \text{if the } i\text{th forward reaction step carries an isotopomer with index } k \\ & \text{over to the isotopomer with index } j \\ -1 & \text{if } j = k \text{ and the } i\text{th forward reaction step} \\ & \text{carries isotopomers away from the pool with index } j \\ 0 & \text{else} \end{cases} \qquad (22)$$

The other matrices $\overline{\mathbf{Q}}_{i,j}^{\leftarrow}, \overline{\mathbf{P}}_i^{\leftarrow}, \overline{\mathbf{P}}_i^{\text{inp}}$ are defined completely analogous.

The same procedure can now be carried out for the cumomer labeling balances. To this end, the weight of an index i within the vector $\mathbf{x}$ is defined as the weight of the corresponding isotopomer or cumomer. Now bearing in mind that the cumomer balances are weight-preserving the procedure (12) can be immediately translated into the formal definition

$$\begin{aligned} (\mathbf{Q}_{i,j}^{\rightarrow})_{k,l} &= \begin{cases} (\overline{\mathbf{Q}}_{i,j}^{\rightarrow})_{k,l} & \text{if weight}\,(k) + \text{weight}\,(l) = \text{weight}\,(j) \\ 0 & \text{else} \end{cases} \\ (\mathbf{P}_i^{\rightarrow})_{k,l} &= \begin{cases} (\overline{\mathbf{P}}_i^{\rightarrow})_{k,l} & \text{if weight}\,(k) = \text{weight}\,(l) \\ 0 & \text{else} \end{cases} \end{aligned} \qquad (23)$$

and the general balance equation then has the same structure as Equation (20) with the bars removed:

$$\frac{1}{2}\,\mathbf{x}^T \cdot \left(\sum_i \mathbf{v}_i^{\rightarrow} \cdot \mathbf{Q}_i^{\rightarrow} + \mathbf{v}_i^{\leftarrow} \cdot \mathbf{Q}_i^{\leftarrow}\right) \cdot \mathbf{x} + \left(\sum_i \mathbf{v}_i^{\rightarrow} \cdot \mathbf{P}_i^{\rightarrow} + \mathbf{v}_i^{\leftarrow} \cdot \mathbf{P}_i^{\leftarrow}\right) \cdot \mathbf{x} + \left(\sum_i \mathbf{v}_i^{\rightarrow} \cdot \mathbf{P}_i^{\text{inp}}\right) \cdot \mathbf{x}^{\text{inp}} = \mathbf{0} \qquad (24)$$

4.4 *Transforming isotopomer into cumomer fractions*

Based on Equation (24) the cumomer fractions $\mathbf{x}$ can be computed by using matrix calculus as is explained in the Appendix. Finally, it must be explained how the isotopomer fractions $\overline{\mathbf{x}}$ (if required) can be obtained from the cumomer fractions. To this end the transformation Equations (7-9) are brought into a general matrix notation. It can be shown that for a single metabolite with n carbon atoms the transformation from its 2^n isotopomer fractions into the corresponding 2^n cumomer fractions is given by the recursively defined square matrices

$$\mathbf{T}_0 = (1)\,, \qquad \mathbf{T}_{n+1} = \begin{pmatrix} \mathbf{T}_n & \mathbf{T}_n \\ \mathbf{0} & \mathbf{T}_n \end{pmatrix}$$

where $\mathbf{0}$ denotes the zero matrix. For example it holds:

$$\begin{pmatrix} d_{xxx} \\ d_{xx1} \\ d_{x1x} \\ d_{x11} \\ d_{1xx} \\ d_{1x1} \\ d_{11x} \\ d_{111} \end{pmatrix} = \underbrace{\left(\begin{array}{cccc|cccc} 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 \\ . & 1 & . & 1 & . & 1 & . & 1 \\ . & . & 1 & 1 & . & . & 1 & 1 \\ . & . & . & 1 & . & . & . & 1 \\ \hline . & . & . & . & 1 & 1 & 1 & 1 \\ . & . & . & . & . & 1 & . & 1 \\ . & . & . & . & . & . & 1 & 1 \\ . & . & . & . & . & . & . & 1 \end{array}\right)}_{\mathbf{T}_3} \cdot \begin{pmatrix} d_{000} \\ d_{001} \\ d_{010} \\ d_{011} \\ d_{100} \\ d_{101} \\ d_{110} \\ d_{111} \end{pmatrix}$$

Consequently, there is an overall block diagonal transformation

$$\mathbf{x} = \begin{pmatrix} \mathbf{T}_{n_1} & \mathbf{0} & \cdots \\ \mathbf{0} & \mathbf{T}_{n_2} & \cdots \\ \vdots & \vdots & \ddots \end{pmatrix} \cdot \overline{\mathbf{x}} \stackrel{def}{=} \mathbf{T} \cdot \overline{\mathbf{x}} \tag{25}$$

where $n_1, n_2, \ldots$ are the numbers of carbon atoms of all intracellular metabolites in the system. Similarly, there is a block diagonal transformation

$$\mathbf{x}^{\text{inp}} = \mathbf{T}^{\text{inp}} \cdot \overline{\mathbf{x}}^{\text{inp}} \quad . \tag{26}$$

It can be easily proven that the inverse of $\mathbf{T}_n$ is recursively given by

$$\mathbf{T}_0^{-1} = (1)\,, \qquad \mathbf{T}_{n+1}^{-1} = \begin{pmatrix} \mathbf{T}_n^{-1} & -\mathbf{T}_n^{-1} \\ \mathbf{0} & \mathbf{T}_n^{-1} \end{pmatrix} \quad . \tag{27}$$

For example it holds:

$$\begin{pmatrix} d_{000} \\ d_{001} \\ d_{010} \\ d_{011} \\ d_{100} \\ d_{101} \\ d_{110} \\ d_{111} \end{pmatrix} = \underbrace{\left(\begin{array}{cccc|cccc} 1 & -1 & -1 & 1 & -1 & 1 & 1 & -1 \\ . & 1 & . & -1 & . & -1 & . & 1 \\ . & . & 1 & -1 & . & . & -1 & 1 \\ . & . & . & 1 & . & . & . & -1 \\ \hline . & . & . & . & 1 & -1 & -1 & 1 \\ . & . & . & . & . & 1 & . & -1 \\ . & . & . & . & . & . & 1 & -1 \\ . & . & . & . & . & . & . & 1 \end{array}\right)}_{\mathbf{T}_3^{-1}} \cdot \begin{pmatrix} d_{xxx} \\ d_{xx1} \\ d_{x1x} \\ d_{x11} \\ d_{1xx} \\ d_{1x1} \\ d_{11x} \\ d_{111} \end{pmatrix}$$

Using these relations the inverse matrices $\mathbf{T}^{-1}, (\mathbf{T}^{\text{inp}})^{-1}$ can be easily computed from Equation (25) so that the switching between the coordinate systems poses no problem.

4.5 *The central theorem for cumomer systems*

The main theorem for the structural analysis of isotopomer labeling systems is the formal statement that the procedure (12) is correct:

> **Theorem:** $\overline{\mathbf{x}}$ is a solution of the isotopomer balances from Equation (20) with input vector $\overline{\mathbf{x}}^{\text{inp}}$ *if and only if* $\mathbf{x} = \mathbf{T}\,\overline{\mathbf{x}}$ is a solution of the cumomer balances from Equation (24) with input vector $\mathbf{x}^{\text{inp}} = \mathbf{T}^{\text{inp}}\,\overline{\mathbf{x}}^{\text{inp}}$.

The rather technical general proof is given in (Wurzel, 1997). Based on this theorem the cumomer fractions can be explicitly computed by successively solving the linear equation systems for the 0-,1-,2-,... cumomer fractions as has been demonstrated for the example from Figure 2. As a consequence the cumomer fractions are always uniquely given as a rational function $\mathbf{\Gamma}$ of the flux vectors $\mathbf{v}^{\rightarrow}, \mathbf{v}^{\leftarrow}$. The same holds for the isotopomer fractions as is shown by using the transformation from Equation (25). This finally generalizes the complete theory developed for positional labeling systems in Part I. Several computational examples will be presented in Part IV.

5 Cumomer Networks

The *isotopomer network* corresponding to a certain *metabolite network* consists of all isotopomers in the system and the reaction steps between them. For example the backward direction of the bimolecular step v3: B + E > C in Figure 2 gives rise to the $2^2 \cdot 2^2 = 16$ isotopomer reactions

v3: C#ijkl > B#ij + E#kl , i,j,k,l = 0,1 .

Since v3 is bidirectional all corresponding isotopomer reactions are bidirectional, too. The isotopomer balance equations can be directly constructed from the isotopomer network as has been explained before. On the other hand, the isotopomer network can be interpreted as a graphical representation of the isotopomer balance equations. Such a graphical representation can be extremely helpful to understand the structural properties of the system and to perform simplification operations (Reddy *et al.*, 1993). For this purpose a *cumomer network* is now constructed in a completely analogous way as a graphical representation of the cumomer balances. This completes the diagram:

	isotopomer network	$\longrightarrow$ transformation	cumomer network	
formulation rules	$\downarrow$		$\downarrow$	formulation rules
	isotopomer balances	$\longrightarrow$ transformation	cumomer balances	

5.1 *Constructing the cumomer network*

The cumomer network is constructed according to the following rules starting with the given isotopomer network. These rules are simply a graphical representation of the procedure (12). Note that for bimolecular steps the forward reaction must be treated differently from the backward reactions:

CN0: Replace each bi-bi-reaction step w: M + N > P + Q by a sequence w1: M + N > MN, w2: MN > P + Q of two reactions which are bimolecular on only one side. It should be mentioned that this step is not really necessary but simplifies the following explanations.

CN1: Replace all isotopomers in the isotopomer network by their corresponding cumomers (i.e. replace each index 0 by X). The result for the backward step of v3 in the running example is:

v3: C#ijkl > B#ij + E#kl, i,j,k,l = X,1

CN2: Remove all 0-cumomers from the network because the corresponding cumomer labeling fractions are 1 and thus do not contribute to the balance equations. Because the reaction v3: C#XXXX > B#XX + E#XX is completely eliminated by this rule the 16 backward reactions of v3 in the example reduce to the following 15 reactions :

```
v3:  C#ijkl  >  B#ij  +  E#kl  , ij=X1,1X,11,  kl =X1,1X,11
v3:  C#XXkl  >           E#kl  ,               kl =X1,1X,11
v3:  C#ijXX  >  B#ij           , ij=X1,1X,11
```

CN3: Remove all reactions with two products that both have positive weight and replace them by a system efflux. Of 15 backward isotopomer reactions of v3 only 6 reactions are thus kept in the cumomer network

```
v3:  C#1XXX  >  B#1X        v3:  C#X1XX  >  B#X1
v3:  C#XX1X  >  E#1X        v3:  C#XXX1  >  E#X1
v3:  C#11XX  >  B#11        v3:  C#XX11  >  E#11
```

while the others are replaced by an efflux:

```
v3:  C#1X1X  >        v3:  C#X1X1  >        v3:  C#1X11  >
v3:  C#1XX1  >        v3:  C#111X  >        v3:  C#X111  >
v3:  C#X11X  >        v3:  C#11X1  >        v3:  C#1111  >
```

The last rule is the most important one because it enforces weight conservation in the cumomer network. No cumomer pool can have an influx from another pool with higher weight. Consequently, if a cumomer is splitted into two products then one of the products must have weight 0 and thus is ommitted by rule CN2.

5.2 *The cascade of cumomer subnetworks*

The resulting cumomer network has considerably fewer bimolecular reaction steps than the isotopomer network because only those bimolecular steps "survive" that combine two cumomers to a product of higher weight. This immediately induces a cascaded structure of the cumomer network that is illustrated in Figure 3:

1. The nodes and edges of the n-cumomer network are all the n-cumomers and the cumomer reaction steps which take place between n-cumomers. These steps are always unimolecular by construction. It is helpful to arrange the cumomer nets in a three-dimensional graphical representation where the $n+1$-cumomer net lies "above" the n-cumomer net (Figure 3).

2. The different n-cumomer networks are "vertically" linked by all the bimolecular reaction steps. Each of these steps by construction combines two cumomers of weight k and l to a cumomer of weight $k+l$, i.e. the bimolecular steps are always directed "upwards" in the graphical representation (Figure 3).

By construction the sum terms in the cumomer balance equations are in a one-to-one correspondence with the reaction arrows in the cumomer network. In the same way, the cascaded network structure directly corresponds to the cascaded linear equation systems presented in Equations (14-18) for the example.

5.3 *An alternative notation*

In order to obtain an easily readable visual representation of the n-cumomer networks an alternative notation for cumomers and cumomer fractions is now introduced. This *positional* notation is much shorter than the binary notation used before. On the other hand, it is not well suited for the formulation of general balance equations like that in Equations (6,13). In the positional cumomer notation only the always labeled positions of a molecule are given by their positional number. For example:

C =C#XXXX	C#24 =C#X1X1	C#1234=C#1111
C#2=C#X1XX	C#234=C#X111	

In the same way the cumomer fractions are denoted by c, c_{2}, c_{24} and so on. Note that $c = 1$ and that in the case of a 1-cumomer this yields exactly the former notation of carbon atoms and positional labeling fractions. So the positional notation is compatible with that introduced in Part I. On the other hand it should not be confused with the positional isotopomer notation used in former publications (Chance *et al.*, 1983). Using the positional notation the example of n-cumomer networks is visualized by a cascade of subnets in Figure 4.

5.4 *The paradox of vanishing cumomers*

There is one paradoxical feature of cumomer networks related to those bimolecular reaction steps with two products. This is the apparent vanishing of cumomers from the system as induced by the rule CN3. For instance, the bimolecular isotopomer reaction step v3 in the example network induces the reaction step

$$\text{v3: B\#2 + E\#12} > \text{C\#234} \quad .$$

in the cumomer network. This step is a transition step from the 1- and 2- cumomer networks to the 3-cumomer network. If the reverse reaction step is considered there only remains an efflux

$$\text{v3: C\#234} >$$

from the system because no reaction step can proceed "downwards" in the cascaded reaction system by rule CN3. Thus C#234 seems to vanish from the system instead of splitting into B#2 and E#12. The explanation for this effect is that the cumomer C#234 – considered as a set of isotopomers – is actually *contained* in both cumomers C#2 and C#34. But the latter have already been taken into account on levels 1 and 2 which explains the paradox:

$$\begin{array}{lll} \text{v3:} & \text{C\#2} & > \text{B\#2} \\ \text{v3:} & \text{C\#34} & > \text{E\#12} \end{array}$$

6 Solving Structural Flux Identifiability Problems

This section is concerned with flux identifiability by isotopomer labeling experiments. The question is whether there is enough information contained in the cumomer labeling fractions to identify all the free fluxes in the system. If this is not the case it is desirable to know which subset of fluxes can be identified. In particular it is of great interest if more flux information can be obtained with isotopomer measurements compared to only positional enrichment measurements. As will be shown, a graphical analysis of the cumomer network helps to eliminate these problems.

The kind of identifiability analysis presented here relies on the assumption that all cumomer fractions are potentially measurable and measurement errors are negligible. Since this is a rather optimistic assumption all results will be best case results, i.e. in the practical experiment even fewer fluxes might be identified. However, the results will not be that far from the real situation because for metabolites with at most three carbon atoms all cumomer fractions can currently be determined by a combination of the different measurement techniques (Wiechert & de Graaf, 1996). More results on the achievable flux information that also take the available measurements and the statistical aspects into account will be presented in Part IV.

6.1 *Simplifying cumomer networks*

As a first step in this analysis the cumomer network is reduced to a simpler one by removing certain nodes. Consider for instance the cumomers of C and D in the example network from Figure 2. Because v6 and v7 are assumed to be unidirectional (an extracellular metabolite splits off) it follows immediately from the cumomer balance Equations (13) and Equation (2) that (using binary notation)

$$d_{ijk} = c_{xijk}, \quad i,j,k = x,1 \qquad \text{and} \qquad c_{ijkl} = b_{ij}\, e_{kl}, \quad i,j,k,l = x,1 \tag{28}$$

(see also Equations (14-18)). Such relations are called labeling *redundancies*.

These redundancies imply that the cumomer fractions of C and D contain no additional flux information as compared to their predecessors B and E. In particular this redundancy holds independently of the current flux values. Thus, with respect to flux identification, redundant variables can be eliminated from the balance equations.

An even better idea is to remove the redundant nodes directly from the cumomer network. To this end, the following graphical elimination rules for cumomer network simplification by removing redundant nodes are generally valid (see Figure 5):

S1: If a metabolite M has only one influx v: N $>$ M and v is unidirectional then M can be removed from the network and within all its (necessarily unidirectional) effluxes w1: M $>$ P1, w2: M $>$ P2,... the node M can be replaced by N.

S2: If a metabolite M has only one influx v: N $>$ M and v is bidirectional then this flux can be assumed to be unidirectional. Its value must be assigned to the net flux of the original step.

By iteratively applying these rules the 3- and 4-nets from Figure 4 completely vanish. Figure 6 shows what results for the 1- and 2-cumomer network. From these networks two simplified sets of balance equations for the remaining two intracellular metabolites B and E can be directly read off:

$$\begin{array}{lrlcl}
\mathsf{B} & : & v_2^{\rightarrow} + v_3^{\text{net}} + v_5^{\rightarrow} & = & v_1^{\rightarrow} + v_2^{\leftarrow} \\
\mathsf{B\#1} & : b_1 & (v_2^{\rightarrow} + v_3^{\text{net}} + v_5^{\rightarrow}) & = & a_1\, v_1^{\rightarrow} + e_1\, v_2^{\leftarrow} \\
\mathsf{B\#2} & : b_2 & (v_2^{\rightarrow} + v_3^{\text{net}} + v_5^{\rightarrow}) & = & a_2\, v_1^{\rightarrow} + e_2\, v_2^{\leftarrow} \\
\mathsf{B\#12} & : b_{12} & (v_2^{\rightarrow} + v_3^{\text{net}} + v_5^{\rightarrow}) & = & a_{12}\, v_1^{\rightarrow} + e_{12}\, v_2^{\leftarrow} \\
\mathsf{E} & : & v_2^{\leftarrow} + v_3^{\text{net}} + v_4^{\rightarrow} & = & v_2^{\rightarrow} + v_3^{\text{net}} \\
\mathsf{E\#1} & : e_1 & (v_2^{\leftarrow} + v_3^{\text{net}} + v_4^{\rightarrow}) & = & b_1\, v_2^{\rightarrow} + b_2\, v_3^{\text{net}} \\
\mathsf{E\#2} & : e_2 & (v_2^{\leftarrow} + v_3^{\text{net}} + v_4^{\rightarrow}) & = & b_2\, v_2^{\rightarrow} + e_1\, v_3^{\text{net}} \\
\mathsf{E\#12} & : e_{12} & (v_2^{\leftarrow} + v_3^{\text{net}} + v_4^{\rightarrow}) & = & b_{12}\, v_2^{\rightarrow} + b_2 e_1\, v_3^{\text{net}}
\end{array} \tag{29}$$

As can be seen, only the net flux of v_3^{net} influences the systems labeling state. Consequently, $v_3^{\rightarrow}$ and $v_3^{\leftarrow}$ cannot be distinguished from flux or labeling measurements. Thus v3 can be assumed to be unidirectional without loss of information.

6.2 *Solving the flux identifiability problem*

Taking $v_3^{\leftarrow} = 0$ the aim is now to represent the remaining 4 free fluxes $v_1^{\rightarrow}, v_2^{\rightarrow}, v_2^{\leftarrow}, v_3^{\rightarrow}$ as a function of the 6 cumomer fractions $b_1, b_2, b_{12}, e_1, e_2, e_{12}$. There is a chance to find such functions because the simplified network from Figure 6 contains no more redundant nodes that can be eliminated with the simplification rules. But unfortunately it will be shown now that the reduced net can still be used to derive another type of redundancy relation.

To this end, it should be noticed that all remaining nodes in the reduced network have only two influxes. In this situation the ratio of these influxes can always be directly computed from labeling fractions. For example, looking at the nodes B#1, B#2 and B#12 and using the flux balance for B in Equation (29) it immediately follows that

$$\frac{v_1^{\rightarrow}}{v_2^{\leftarrow}} = -\frac{e_1 - b_1}{a_1 - b_1} = -\frac{e_2 - b_2}{a_2 - b_2} = -\frac{e_{12} - b_{12}}{a_{12} - b_{12}} \tag{30}$$

which are three different formulas for the same flux ratio. This is an immediate consequence of the fact that the network has the same structure at all three cumomer nodes. Similarly, another flux ratio can be computed threefold from the E balances:

$$\frac{v_2^{\rightarrow}}{v_3^{\rightarrow}} = -\frac{e_1 - b_2}{e_1 - b_1} = -\frac{e_2 - e_1}{e_2 - b_2} = -\frac{e_{12} - e_1 \cdot b_2}{e_{12} - b_{12}} \tag{31}$$

As an immediate consequence there are four algebraic relations from Equations (30,31) between the labeling variables that hold independently from the actual flux situation. These relations can be used to eliminate some

variables from the system. For example it follows from Equation (30) that

$$\begin{aligned} e_2 &= b_2 + \frac{e_1 - b_1}{a_1 - b_1} \cdot (a_2 - b_2) \\ e_{12} &= b_{12} + \frac{e_1 - b_1}{a_1 - b_1} \cdot (a_{12} - b_{12}) \end{aligned}$$

always hold. Substituting these equations into Equation (31) (which is not carried out here for the sake of brevity) it finally turns out that four of the six labeling variables are redundant with the others and thus carry no additional flux information.

In summary, all cumomer labeling fractions in the system can be directly computed from the knowledge of only two cumomer fractions as e.g. b_1, e_1 irrespective of the current flux values. Even if one extracellular flux were directly measured this would not help to determine all the fluxes from whatever cumomer fractions are given. However, partial information can be obtained. If for example $v_1^{\rightarrow}$ is measured then $v_2^{\leftarrow}$ can be determined from Equation (30) while $v_2^{\rightarrow}$ and $v_3^{\rightarrow}$ remain hidden. Finally, if two fluxes like e.g. $v_1^{\rightarrow}$ and $v_6^{\rightarrow}$ are measured all other fluxes can be computed from Equations (30) and (31) by using only fractional enrichment data.

6.3 *Another interesting example*

The example discussed above is somewhat disappointing because in this case isotopomer measurements are not superior to positional measurements. However, this is not generally the case as the example from Figure 7 shows, that is derived from an example in (Schmidt, 1998). It is now rigorously analyzed with the methods presented above. The corresponding reaction equations are given by (see Figure 7):

u:	A >B #xy>#xy	v1:	B >C #xy>#xy	w1:	C >F #xy >#xy
r :	F >G #xy>#xy	v2:	B >E #xy>#yx	w2:	E >F #xy >#xy
		v3:	B >D +D #xy>#x +#y	w3:	D +D >F #x +#y>#xy

The example is constructed such that the reactions v1,w1 and v2,w2 keep the two carbon atoms of the input metabolite A together (but with opposite orientation) while v3 splits the molecule D and w3 reunites the carbon atoms. All fluxes are assumed to be unidirectional and the free fluxes are $v_1^{\rightarrow}, v_2^{\rightarrow}, v_3^{\rightarrow}$. The substrate uptake

$$u^{\rightarrow} = v_1^{\rightarrow} + v_2^{\rightarrow} + v_3^{\rightarrow} \tag{32}$$

is assumed to be measured as usual. The remaining fluxes are then given by $w_i^{\rightarrow} = v_i^{\rightarrow},\ i = 1, 2, 3$ and $r^{\rightarrow} = u^{\rightarrow}$.

From Figure 7 it becomes immediately clear that all nodes are redundant except for D#1, F#1, F#2 and F#12. The reduced 1- and 2-cumomer networks are shown in Figure 8. From these nets the reduced balance equations are given as:

$$\begin{array}{lrcl} \text{D\#1} : & d_1\ 2\,v_3^{\rightarrow} & = & a_1\ v_3^{\rightarrow} + a_2\ v_3^{\rightarrow} \\ \text{F\#1} : & f_1\ (v_1^{\rightarrow} + v_2^{\rightarrow} + v_3^{\rightarrow}) & = & a_1\ v_1^{\rightarrow} + a_2\ v_2^{\rightarrow} + d_1 v_3^{\rightarrow} \\ \text{F\#2} : & f_2\ (v_1^{\rightarrow} + v_2^{\rightarrow} + v_3^{\rightarrow}) & = & a_2\ v_1^{\rightarrow} + a_1\ v_2^{\rightarrow} + d_1 v_3^{\rightarrow} \\ \text{F\#12}: & f_{12}\,(v_1^{\rightarrow} + v_2^{\rightarrow} + v_3^{\rightarrow}) & = & a_{12} v_1^{\rightarrow} + a_{12} v_2^{\rightarrow} + d_1^2 v_3^{\rightarrow} \end{array} \tag{33}$$

The most important structural property of this example is that the D-nodes only occur on level 0 and 1. On level 1 D#1 has two separate influxes and it follows:

$$d_1 = \frac{1}{2}\,(a_1 + a_2) \tag{34}$$

Thus d_1 is a redundant node. Substituting the value for d_1 into the balances for F#1, F#2 and summing up these two balances it turns out that

$$a_1 + a_2 = f_1 + f_2 \quad . \tag{35}$$

This means that f_1 is redundant with f_2 and consequently there is no chance to determine the three free fluxes from positional labeling data even if the influx $u^{\rightarrow}$ is directly measured. Interestingly, by subtracting the balance for F#1 from that for F#2 in Equation (33) it can be seen that

$$v_1^{\rightarrow} - v_2^{\rightarrow} = u^{\rightarrow} \cdot \frac{f_1 - f_2}{a_1 - a_2} \quad , \tag{36}$$

i.e. at least the difference flux can be obtained from the positional enrichment data.

The last hope for flux identification is that f_{12} contains some additional information on the free fluxes. Indeed from the corresponding balance and Equation (34) it follows:

$$v_3^{\rightarrow} = u^{\rightarrow} \cdot \frac{a_{12} - f_{12}}{a_{12} - (a_1 + a_2)^2/4} \tag{37}$$

Combining this result with Equations (32,36) all free fluxes are determined from labeling measurements. Thus an example has been found, where the isotopomer measurements contain *more* information than the positional labeling measurements.

6.4 *The general concepts*

The above results have been derived in a rather intuitive way. However there is a systematic way to produce redundancy relations for labeling fractions like those in Equations (30,31,35) and identifiability relations for fluxes like those in Equations (36,37) by using the computer algebraic algorithms developed in (Wiechert, 1995). However, for ease of understanding these algorithmic details have been omitted in this text.

The most important concept for the analysis of cumomer networks with respect to flux identifiability is the redundancy of cumomer fractions. A (general) *redundancy* is a (possibly nonlinear) equation $f(\mathbf{x}) = 0$ that holds whatever the flux values in the metabolic system are. In this situation one variable $\mathbf{x}_i$ can be expressed by the others and thus contains no additional information on the fluxes. So the presence of redundancies reduces the available information for flux identification. Once all the redundancies have been determined the flux identifiability can be easily decided based on the dimensional relation:

$$\begin{array}{rcl} \text{Number of identifiable free fluxes} & \leq & \text{Number of independently measured fluxes} \\ & + & \text{Number of isotopomer measurements} \\ & - & \text{Number of redundant isotopomer fractions} \end{array} \tag{38}$$

However, it must be pointed out that the number of redundant fractions may not equal the number of redundancy relations found. The reason is that there may be complex algebraic dependencies between the relations that are hard to find in general (Cox *et al.*, 1992). Fortunately, the algebraic independency of the few nonlinear equations derived in the examples above can be proven by using the computer algebraic methods from (Cox *et al.*, 1992).

7 Conclusion

In this contribution it has been shown that isotopomer systems have much in common with positional labeling systems. In fact they are not as nonlinear as was previously assumed. After a linear transformation from the isotopomer space to the cumomer space the balances can be solved from a cascade of linear equations. In particular the balances for metabolite fluxes, positional carbon enrichments and isotopomer fractions are just three facets of one unifying cumomer balance equation.

For this reason cumomer systems now seem to be the more adequate representation of the balances because the system can be better understood by using these coordinates. The greater simplicity of the cumomer balances is reflected by the cascaded structure of the cumomer networks which contain much fewer bimolecular steps. All numerical and statistical methods formerly derived for positional labeling systems can now be extended straightforwardly to isotopomer systems. This will be carried out in Part IV.

A simple example showed that – compared to positional labeling systems – isotopomer measurements in principle enable additional fluxes to be determined. On the other hand, the given examples of cumomer systems lead to the conjecture that the achievable information increase is not as large as might be expected from the sheer number of available measurements. In particular, the structure of the higher cumomer networks becomes progressively simpler. For example all 5-, 6- and 7-cumomer nodes and almost all 4-cumomer nodes in the pentose phosphate pathway are isolated nodes, i.e. their labeling state is completely determined by the labeling state of the lower cumomer nets. From these considerations it can be conjectured that the carbon atom network is generally the most informative part of the network and the higher networks contain progressively less flux information due to redundancy.

A clear statement can be made concerning labeling experiments with fully labeled substrates (Szyperski, 1995). For such experiments it is clear that all positional enrichments in the system will always become equal to the fraction of fully labeled molecules in the input. Thus the carbon atom network contains absolutely no information for such experiments and all fluxes have to be computed from higher cumomer measurements. Clearly, this approach will be suboptimal (which has also been found by (Schmidt, 1998)) and will be quantitatively demonstrated in Part IV. A better approach might be to apply a mixture of a completely labeled and a positionally labeled isotopomer as input. This question will be decided in Part IV.

A General Solution of the Cumomer Balance Equations

The solution algorithm for general cumomer balance equations has been introduced by using the example from Figure 2. This algorithm will now be developed in complete generality to enable an automatic solution based on matrix calculus. A complete software implementation of the computational procedures has been supplied by the authors.

A.1 *Weight ordering of the state vector*

The key feature of the cumomer fraction balances turned out to be their weight preservation. In this section another ordering of the vectors $\mathbf{x}^{\text{inp}}, \mathbf{x}$ is used that is more feasible for the exploitation of weight preservation than the binary ordering introduced in the main text. A *weight ordering* of $\mathbf{x}$ first arranges all cumomer fractions by their weight and then orders the cumomers with equal weight binarily. For example a binary variable ordering for the example from Figure 2 is given by (cf. Equation 19)

$$\begin{array}{rll} \mathbf{x} = (& b_{xx}, c_{xxxx}, d_{xxx}, e_{xx}, & (\text{weight } 0) \\ & b_{x1}, b_{1x}, c_{xxx1}, c_{xx1x}, c_{x1xx}, c_{1xxx}, d_{xx1}, d_{x1x}, d_{1xx}, e_{x1}, e_{1x}, & (\text{weight } 1) \\ & b_{11}, c_{xx11}, c_{x1x1}, c_{x11x}, c_{1xx1}, c_{1x1x}, c_{11xx}, d_{x11}, d_{1x1}, d_{11x}, e_{11}, & (\text{weight } 2) \\ & c_{x111}, c_{1x11}, c_{11x1}, c_{111x}, d_{111}, & (\text{weight } 3) \\ & c_{1111})^T & (\text{weight } 4) \end{array} .$$

Using a weight ordering $\mathbf{x}$ and $\mathbf{x}^{\text{inp}}$ can be partitioned as

$$\mathbf{x}^{\text{inp}} = \begin{pmatrix} {}^0\mathbf{x}^{\text{inp}} \\ {}^1\mathbf{x}^{\text{inp}} \\ \vdots \end{pmatrix} \qquad \text{and} \qquad \mathbf{x} = \begin{pmatrix} {}^0\mathbf{x} \\ {}^1\mathbf{x} \\ \vdots \end{pmatrix}$$

where the vectors ${}^k\mathbf{x}^{\text{inp}}, {}^k\mathbf{x}$ comprise all cumomer fractions $\mathbf{x}_i$ with weight $(i) = k$. It should be noted that there is a change in the meaning of the state vectors $\mathbf{x}^{\text{inp}}, \mathbf{x}$ compared to their definition in Part I. The former positional labeling state vectors are exactly ${}^1\mathbf{x}^{\text{inp}}, {}^1\mathbf{x}$, i.e. a segment of the new state vectors. Clearly, the defining Equations (21,22, 23) produce different transition matrices for different orderings of the state vector. However, Equation (24) remains correct for any ordering because vectors and matrices are permuted in the same way.

This does not hold for the transformation from isotopomer into cumomer fractions as given in Equation (26) because this definition depends on a binary index ordering. If a weight ordering is chosen for $\mathbf{x}^{\text{inp}}, \mathbf{x}$ then before applying the transformation matrix from Equation (25) all entries of the state vectors must be first reordered into a binary ordering. This is achieved by using permutation matrices $\mathbf{\Pi}^{\text{inp}}, \mathbf{\Pi}$. The general transformation rule then is

$$\mathbf{x}^{\text{inp}} = \mathbf{\Pi}^{\text{inp},T}\, \mathbf{T}^{\text{inp}}\, \mathbf{\Pi}^{\text{inp}} \cdot \overline{\mathbf{x}}^{\text{inp}} \quad \text{and} \quad \mathbf{x} = \mathbf{\Pi}^T\, \mathbf{T}\, \mathbf{\Pi} \cdot \overline{\mathbf{x}}$$

and the inverse transformation is also easy to compute by using Equation (27) and $\mathbf{\Pi}^{-1} = \mathbf{\Pi}^T$ which always holds for permutation matrices.

A.2 *Partitioning of the system matrices*

Corresponding to the partitioning of the state vectors into segments of equal weight in definition (A.1) the unimolecular transition matrices $\mathbf{P}_i^{\rightarrow}$ can be partitioned into a block diagonal structure as

$$\mathbf{P}_i^{\rightarrow} = \begin{pmatrix} {}^0\mathbf{Q}_i^{\rightarrow} & \mathbf{0} & \cdots & \mathbf{0} \\ \mathbf{0} & {}^1\mathbf{Q}_i^{\rightarrow} & \cdots & \mathbf{0} \\ \vdots & \vdots & \ddots & \vdots \end{pmatrix}$$

with $\dim {}^j\mathbf{x} \times \dim {}^j\mathbf{x}$-matrices ${}^j\mathbf{Q}_i^{\rightarrow}$. The reason for the diagonal shape is that due to weight preservation a cumomer fraction can only contribute to the balance of a cumomer with identical weight. Consequently,

$$\mathbf{P}_i^{\rightarrow} \cdot \mathbf{x} = \begin{pmatrix} {}^0\mathbf{P}_i^{\rightarrow} \cdot {}^0\mathbf{x} \\ {}^1\mathbf{P}_i^{\rightarrow} \cdot {}^1\mathbf{x} \\ {}^2\mathbf{P}_i^{\rightarrow} \cdot {}^2\mathbf{x} \\ \vdots \end{pmatrix} . \tag{39}$$

Similar formulas hold for $\mathbf{P}_i^{\leftarrow} \cdot \mathbf{x}$ and $\mathbf{P}_i^{\text{inp}} \cdot \mathbf{x}^{\text{inp}}$.

In the same way, the 3-dimensional bimolecular transition matrices $\mathbf{Q}_i^{\rightarrow}$ partition into $\dim {}^j\mathbf{x} \times \dim {}^k\mathbf{x} \times \dim {}^l\mathbf{x}$ submatrices but only those submatrices with $j = k+l$ are nonzero. These nonzero submatrices are denoted by ${}^{k,l}\mathbf{Q}_i^{\rightarrow}$, which contains all bimolecular transitions where a k-cumomer fraction combines with an l-cumomer fraction thus contributing to the balance of a $k+l$- cumomer fraction. This yields the formula

$$\mathbf{x}^T \cdot \mathbf{Q}_i^{\rightarrow} \cdot \mathbf{x} = \begin{pmatrix} {}^0\mathbf{x}^T \cdot {}^{0,0}\mathbf{Q}_i^{\rightarrow} \cdot {}^0\mathbf{x} \\ {}^0\mathbf{x}^T \cdot {}^{0,1}\mathbf{Q}_i^{\rightarrow} \cdot {}^1\mathbf{x} + {}^1\mathbf{x}^T \cdot {}^{1,0}\mathbf{Q}_i^{\rightarrow} \cdot {}^0\mathbf{x} \\ {}^0\mathbf{x}^T \cdot {}^{0,2}\mathbf{Q}_i^{\rightarrow} \cdot {}^2\mathbf{x} + {}^1\mathbf{x}^T \cdot {}^{1,1}\mathbf{Q}_i^{\rightarrow} \cdot {}^1\mathbf{x} + {}^2\mathbf{x}^T \cdot {}^{0,2}\mathbf{Q}_i^{\rightarrow} \cdot {}^2\mathbf{x} \\ \vdots \end{pmatrix}$$

and a similar one for $\mathbf{x}^T \cdot \mathbf{Q}_i^{\leftarrow} \cdot \mathbf{x}$.

A.3 *General solution of the balance equations*

With these matrix partitions the general algorithm for solving the cumomer balance equations can now be given. It starts with ${}^0\mathbf{x} = \mathbf{1}$ (the vector composed of all 1s) and continues recursively with the solution for ${}^1\mathbf{x}, {}^2\mathbf{x}, \ldots$. To this end, it is now assumed that all cumomer fractions ${}^0\mathbf{x}, {}^1\mathbf{x}, \ldots, {}^{n-1}\mathbf{x}$ have already been computed. Then from Equations (24,39), the balances for the n-cumomer fractions can be written as:

$$\begin{aligned} &\frac{1}{2} \sum_{k+l=n} \left[{}^k\mathbf{x}^T \cdot \left(\sum_i \mathbf{v}_i^{\rightarrow} \cdot {}^{k,l}\mathbf{Q}_i^{\rightarrow} + \mathbf{v}_i^{\leftarrow} \cdot {}^{k,l}\mathbf{Q}_i^{\leftarrow} \right) \cdot {}^l\mathbf{x} \right] \\ &\quad + \left(\sum_i \mathbf{v}_i^{\rightarrow} \cdot {}^n\mathbf{P}_i^{\rightarrow} + \mathbf{v}_i^{\leftarrow} \cdot {}^n\mathbf{P}_i^{\leftarrow} \right) \cdot {}^n\mathbf{x} + \left(\sum_i \mathbf{v}_i^{\rightarrow} \cdot {}^n\mathbf{P}_i^{\text{inp}} \right) \cdot {}^n\mathbf{x}^{\text{inp}} = \mathbf{0} \,. \end{aligned} \tag{40}$$

Now from the symmetry of $\mathbf{Q}_i^{\rightarrow}, \mathbf{Q}_i^{\leftarrow}$ with respect to $\mathbf{x}$ it follows

$${}^0\mathbf{x}^T \cdot {}^{0,n}\mathbf{Q}_i \cdot {}^n\mathbf{x} = \mathbf{1} \cdot {}^{0,n}\mathbf{Q}_i \cdot {}^n\mathbf{x} \quad \text{and also} \quad {}^n\mathbf{x} \cdot {}^{n,0}\mathbf{Q}_i \cdot {}^0\mathbf{x} = \mathbf{1} \cdot {}^{n,0}\mathbf{Q}_i \cdot {}^n\mathbf{x} \quad .$$

Here $\mathbf{1} \cdot {}^{0,n}\mathbf{Q}_i$ and $\mathbf{1} \cdot {}^{n,0}\mathbf{Q}_i$ are just ordinary matrices so that all terms become linear with respect to ${}^n\mathbf{x}$. Rearranging Equation (40) by exposing ${}^n\mathbf{x}$ now produces:

$$\begin{aligned} &\underbrace{\sum_i \left[\mathbf{v}_i^{\rightarrow} \cdot \mathbf{1} \cdot ({}^{n,0}\mathbf{Q}_i^{\rightarrow} + {}^{0,n}\mathbf{Q}_i^{\rightarrow}) + \mathbf{v}_i^{\leftarrow} \cdot \mathbf{1} \cdot ({}^{n,0}\mathbf{Q}_i^{\leftarrow} + {}^{0,n}\mathbf{Q}_i^{\leftarrow}) + \mathbf{v}_i^{\rightarrow} \cdot {}^n\mathbf{P}_i^{\rightarrow} + \mathbf{v}_i^{\leftarrow} \cdot {}^n\mathbf{P}_i^{\leftarrow} \right]}_{\text{known matrix } {}^n\mathbf{A}(\mathbf{v})} \cdot {}^n\mathbf{x} \\ + &\underbrace{\frac{1}{2} \sum_{\substack{k+l=n \\ k,l \neq 0}} \left[{}^k\mathbf{x}^T \cdot \left(\sum_i \mathbf{v}_i^{\rightarrow} \cdot {}^{k,l}\mathbf{Q}_i^{\rightarrow} + \mathbf{v}_i^{\leftarrow} \cdot {}^{k,l}\mathbf{Q}_i^{\leftarrow} \right) \cdot {}^l\mathbf{x} \right] + \sum_i \left(\mathbf{v}_i^{\rightarrow} \cdot {}^n\mathbf{P}_i^{\text{inp}} \right) \cdot {}^n\mathbf{x}^{\text{inp}}}_{\text{known vector } {}^n\mathbf{b}(\mathbf{v}, {}^1\mathbf{x}, \ldots, {}^{n-1}\mathbf{x})} = \mathbf{0} \,. \end{aligned}$$

From this the solution ${}^n\mathbf{x}$ is computed as

$${}^n\mathbf{x} = {}^n\mathbf{A}^{-1}(\mathbf{v}) \cdot {}^n\mathbf{b}(\mathbf{v}, {}^1\mathbf{x}, \ldots, {}^{n-1}\mathbf{x}) \quad .$$

It can be proven that the matrix ${}^n\mathbf{A}$ is invertible in all practically relevant situations (Wurzel, 1997).

In summary, the vector $\mathbf{x}$ is computed as a function of $\mathbf{x}^{\text{inp}}$ and $\mathbf{v}$ by solving a cascade of linear equations:

$$\begin{aligned} \mathbf{1} &= {}^0\mathbf{x} \\ \mathbf{0} &= {}^1\mathbf{A}(\mathbf{v}) \cdot {}^1\mathbf{x} + {}^1\mathbf{b}(\mathbf{v}) \\ \mathbf{0} &= {}^2\mathbf{A}(\mathbf{v}) \cdot {}^2\mathbf{x} + {}^2\mathbf{b}(\mathbf{v}, {}^1\mathbf{x}) \\ \mathbf{0} &= {}^3\mathbf{A}(\mathbf{v}) \cdot {}^3\mathbf{x} + {}^3\mathbf{b}(\mathbf{v}, {}^1\mathbf{x}, {}^2\mathbf{x}) \\ &\vdots \end{aligned} \tag{41}$$

Here the 1-cumomer equation is exactly the well-known positional carbon labeling balance equation from Part I.

A.4 *Derivative of the balance equations*

Numerical optimization algorithms and the statistical evaluation methods that will be developed in Part IV require the knowledge of the derivative $\partial\mathbf{x}/\partial\mathbf{v}$ (i.e. the sensitivity of the labeling state with respect to the fluxes). The straightforward way to calculate these sensitivities is given by an implicit differentiation of the balance Equation (24). Although this is quite easy to implement it is computationally rather expensive because a matrix of dimension $\dim\ \mathbf{x}$ has to be inverted which requires $O(\dim\ \mathbf{x}^3)$ computational operations.

A much more efficient way is to differentiate the whole cascade (41). At level n an implicit differentiation yields:

$$\mathbf{0} = \frac{\partial({}^n\mathbf{A})}{\partial \mathbf{v}_i^{\rightarrow}}(\mathbf{v}) \cdot {}^n\mathbf{x} + {}^n\mathbf{A}(\mathbf{v}) \cdot \frac{\partial({}^n\mathbf{x})}{\partial \mathbf{v}_i^{\rightarrow}} + \frac{\partial({}^n\mathbf{b})}{\partial \mathbf{v}} \cdot \frac{\partial \mathbf{v}}{\partial \mathbf{v}_i^{\rightarrow}} + \sum_{i=1}^{n-1} \frac{\partial({}^n\mathbf{b})}{\partial {}^i\mathbf{x}} \cdot \frac{\partial({}^i\mathbf{x})}{\partial \mathbf{v}_i^{\rightarrow}} \tag{42}$$

The only unknown quantity at this stage is $\partial({}^n\mathbf{x})/\partial \mathbf{v}_i^{\rightarrow}$ which means that the matrix factorization for ${}^n\mathbf{A}(\mathbf{v})$ that was necessary to solve Equation (41) can be reused for solving Equation (42). Because matrix factorization is the most time consuming operation in the solution algorithm this shows that the sensitivities can be computed with negligible effort. However the proper implementation of the implicit differentiation procedure is rather difficult and must be carefully tested. This has been done by computing numerical derivatives in parallel.

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B Figures and Tables

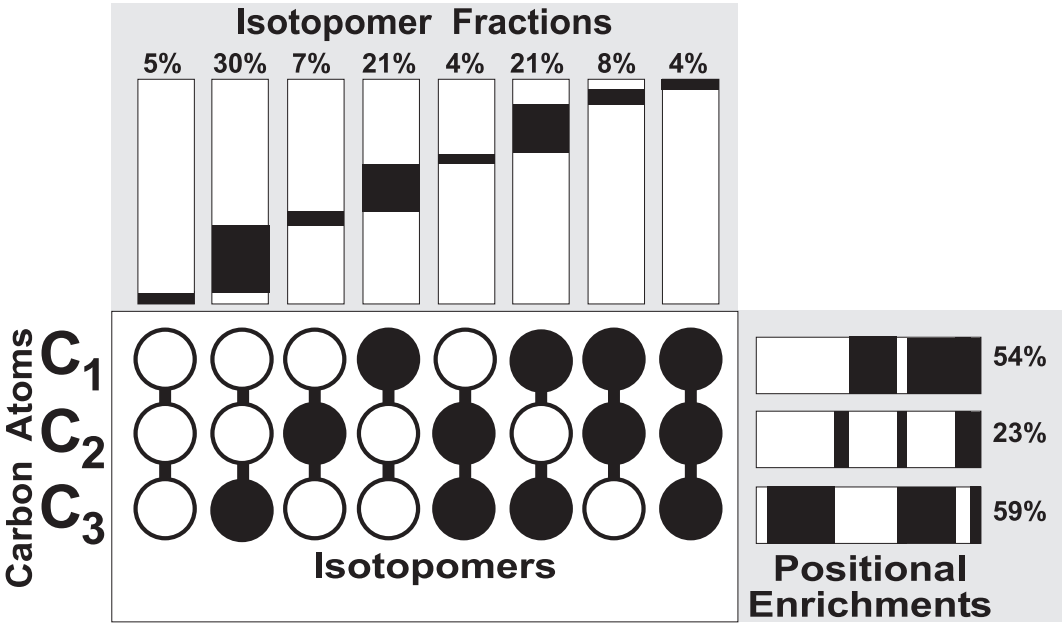

Figure 1: The $2^3 = 8$ isotopomers of a molecule with 3 carbon atoms together with the corresponding isotopomer fractions and positional carbon enrichments

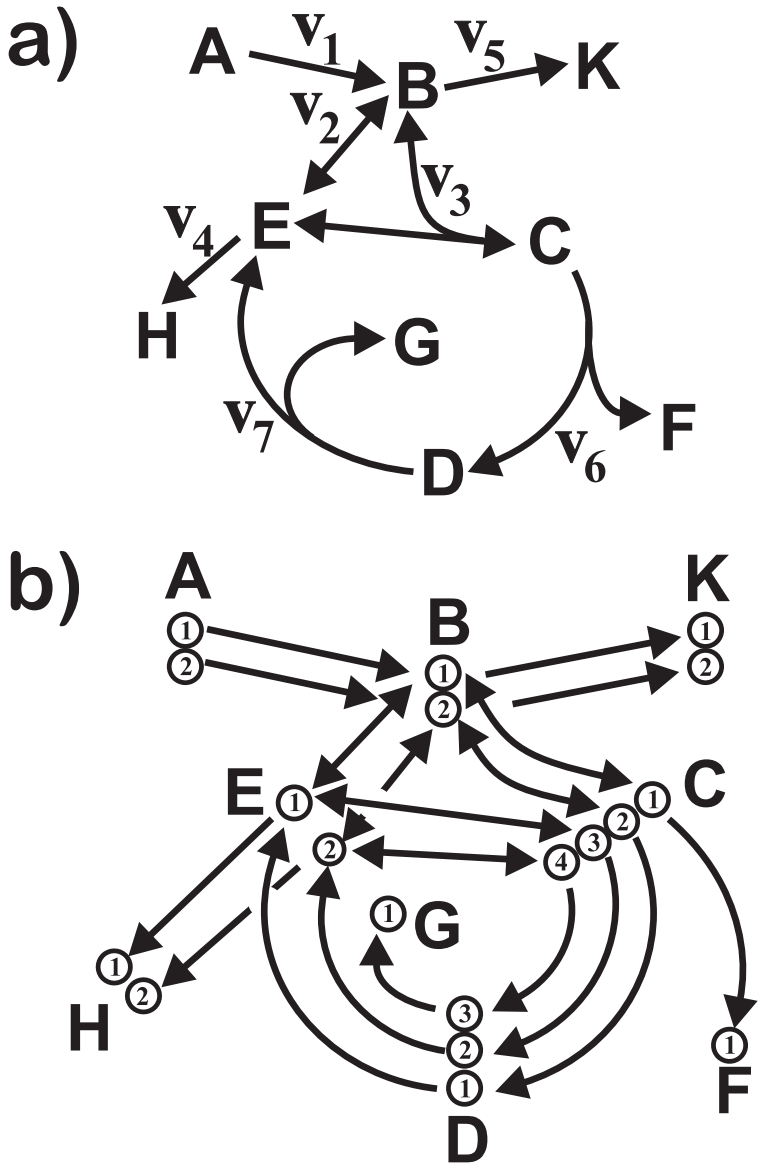

Figure 2: Example network used for the introduction of isotopomer and cumomer balances. a) Metabolic network with flux names. b) Corresponding carbon atom transitions.

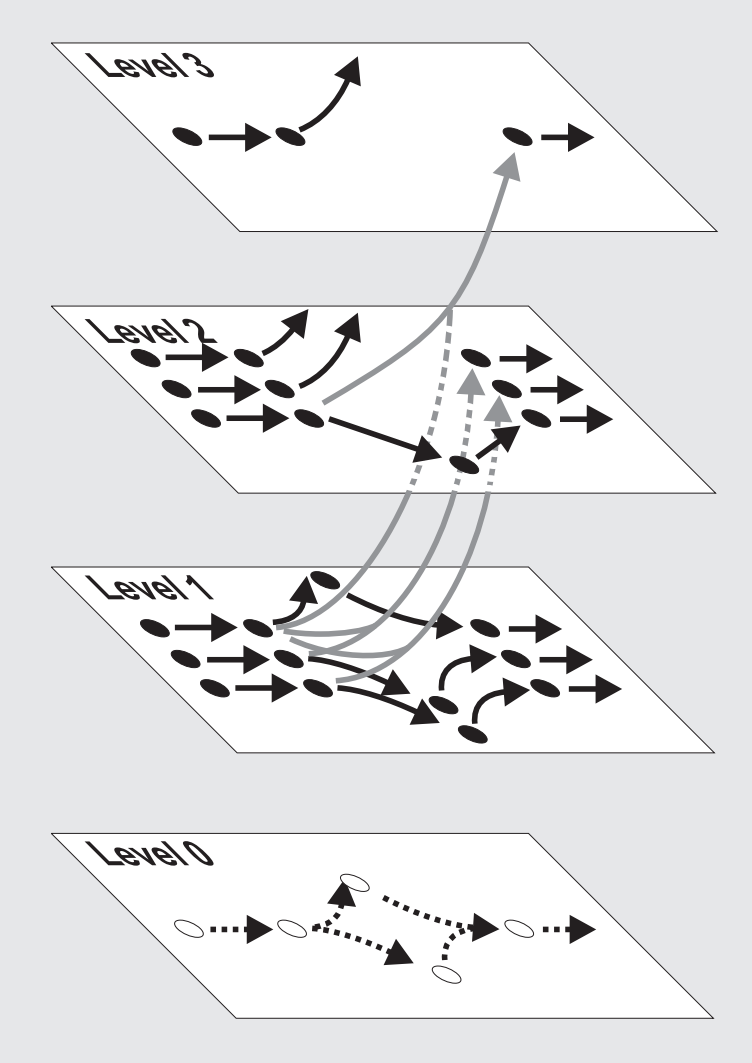

Figure 3: Cascaded structure of the cumomer network. The n-cumomer networks are arranged vertically and they are linked by the bimolecular reaction steps. The 0-cumomer network is identical with the underlying metabolic network and usually is completely eliminated because all its cumomer values are 1 by definition. However it has been kept in the Figure for illustrative purposes.

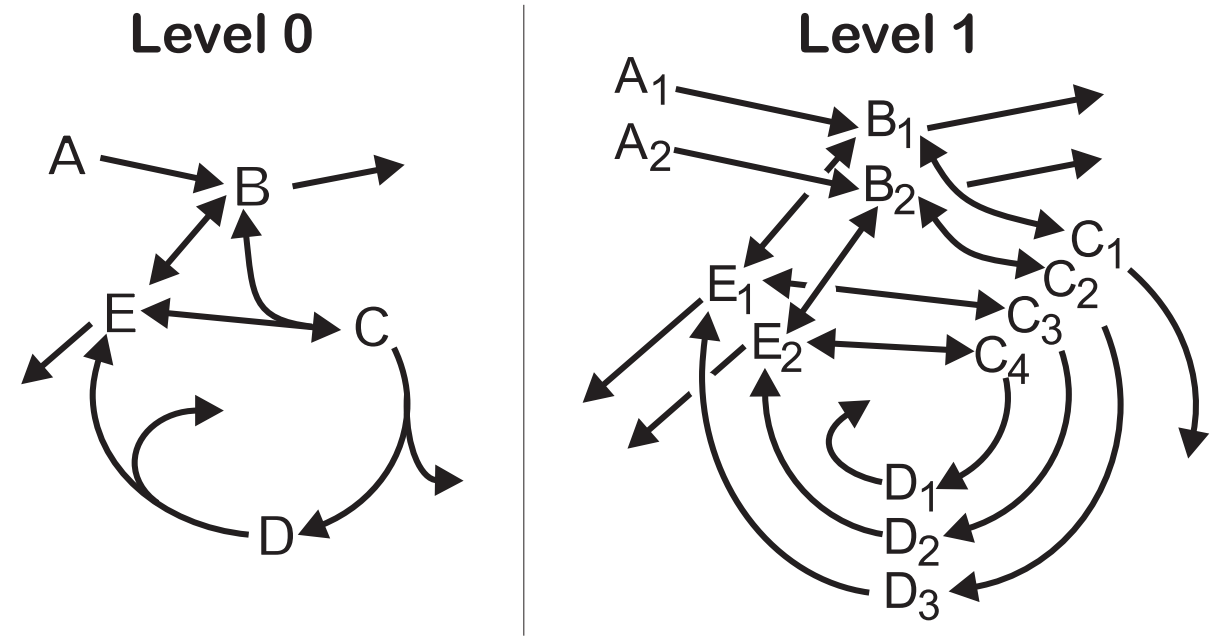

Figure 4: All n-cumomer networks ($n = 0, 1, 2, 3, 4$) for the example from Figure 2. The shaded bimolecular steps are only drawn within their target subnetwork but without their educts, which belong to a lower level.

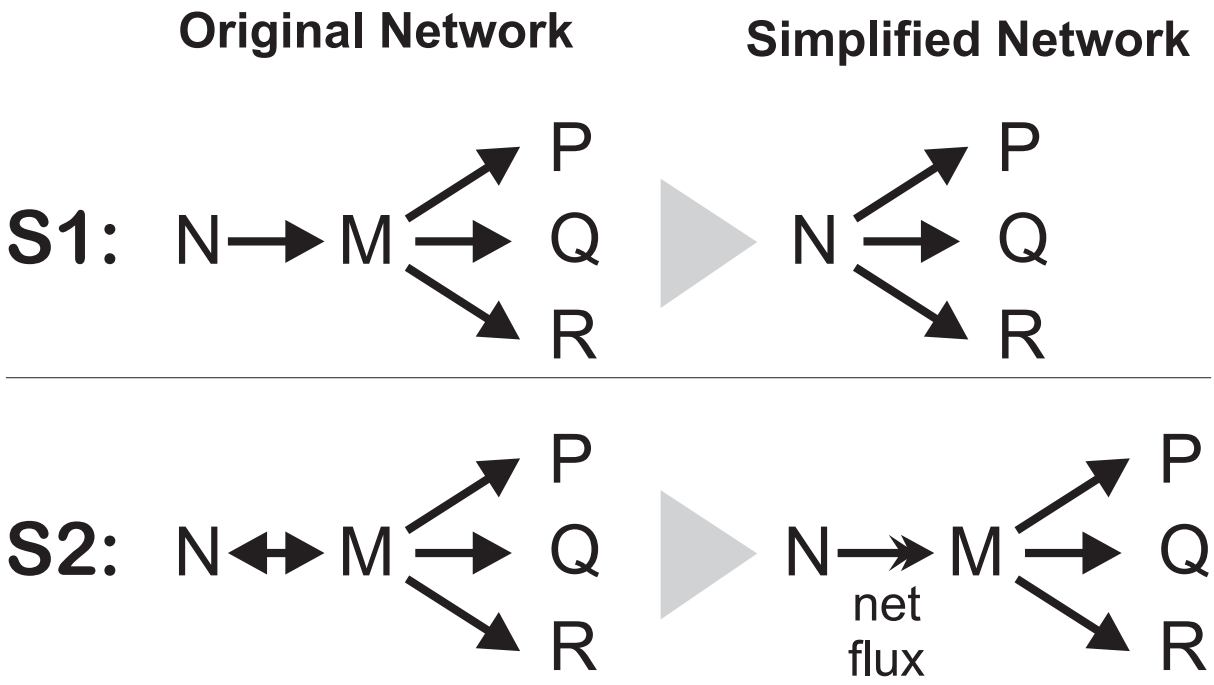

Figure 5: Schematic representation of the network simplification rules for eliminating redundant nodes.

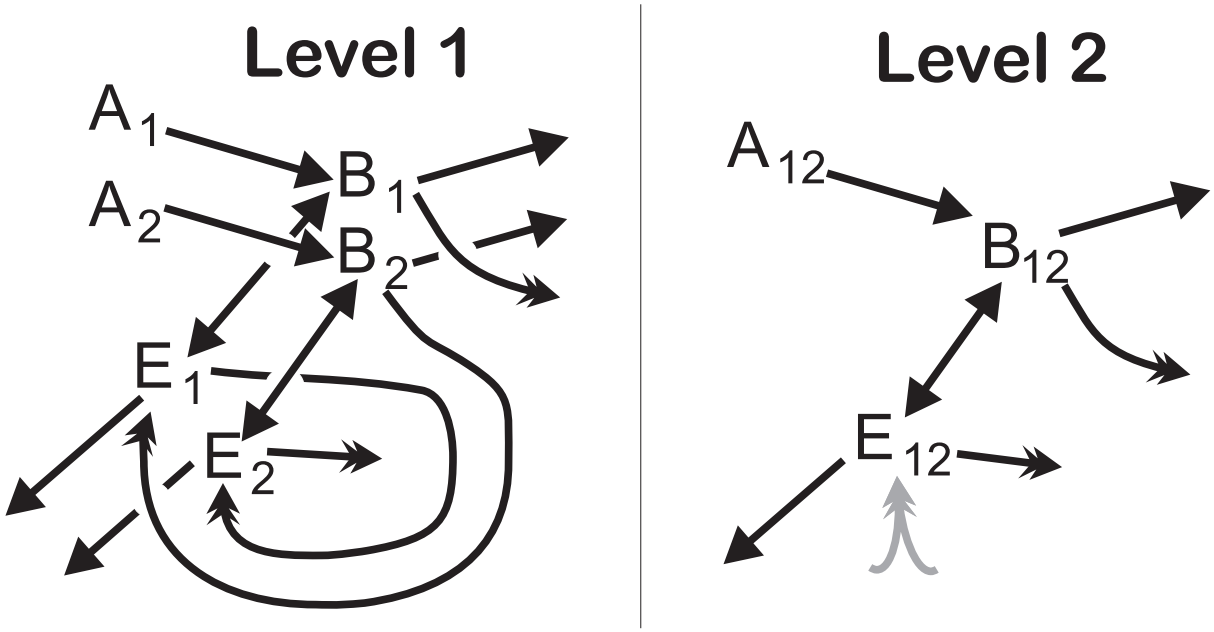

Figure 6: Reduced 1- and 2-cumomer networks of the example network from Figure 4. Bidirectional fluxes that have been replaced by net fluxes (see Figure 5) are indicated by a feathered arrow.

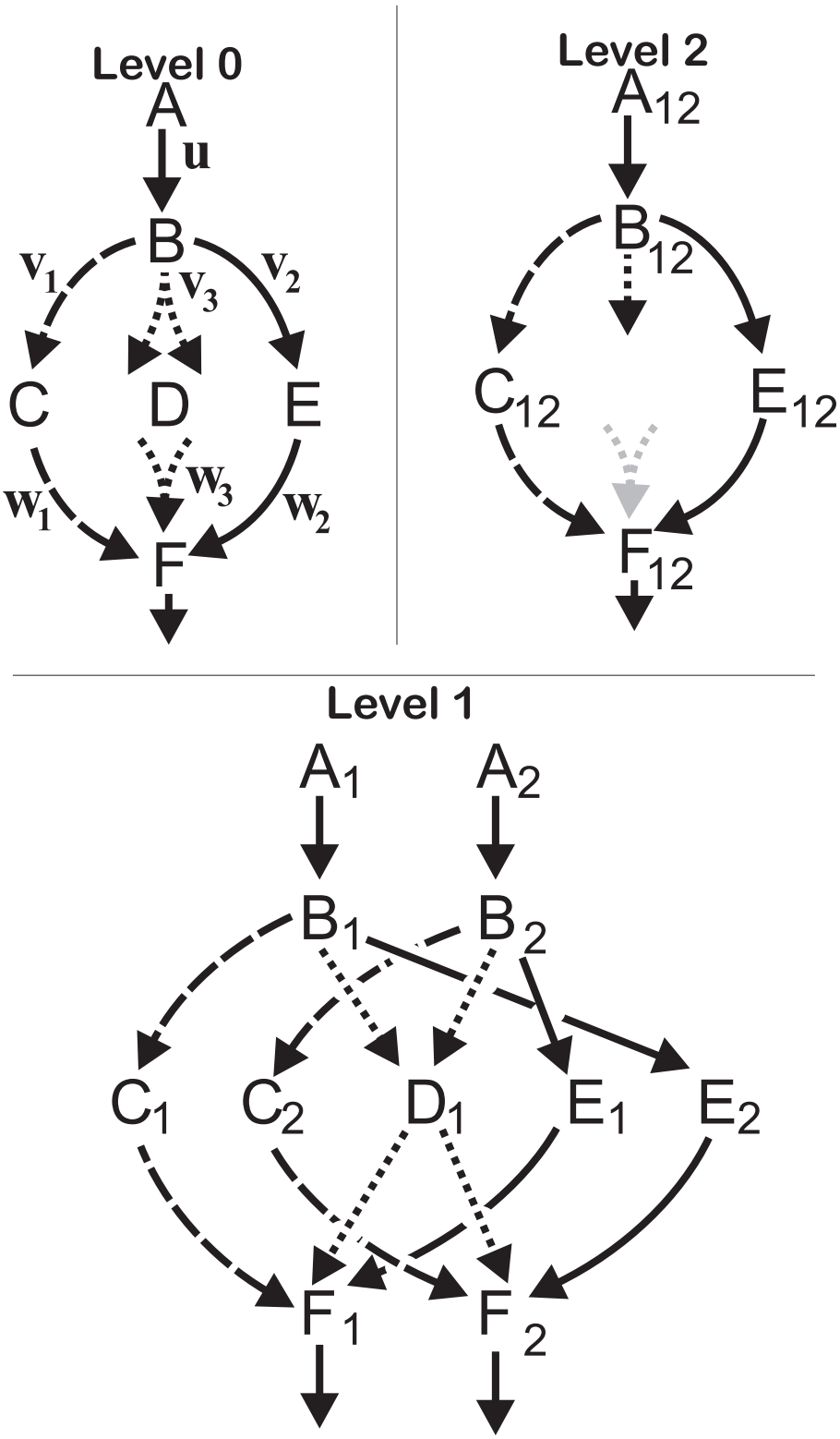

Figure 7: Example network proving the principal superiority of isotopomer experiments over positional enrichment experiments.

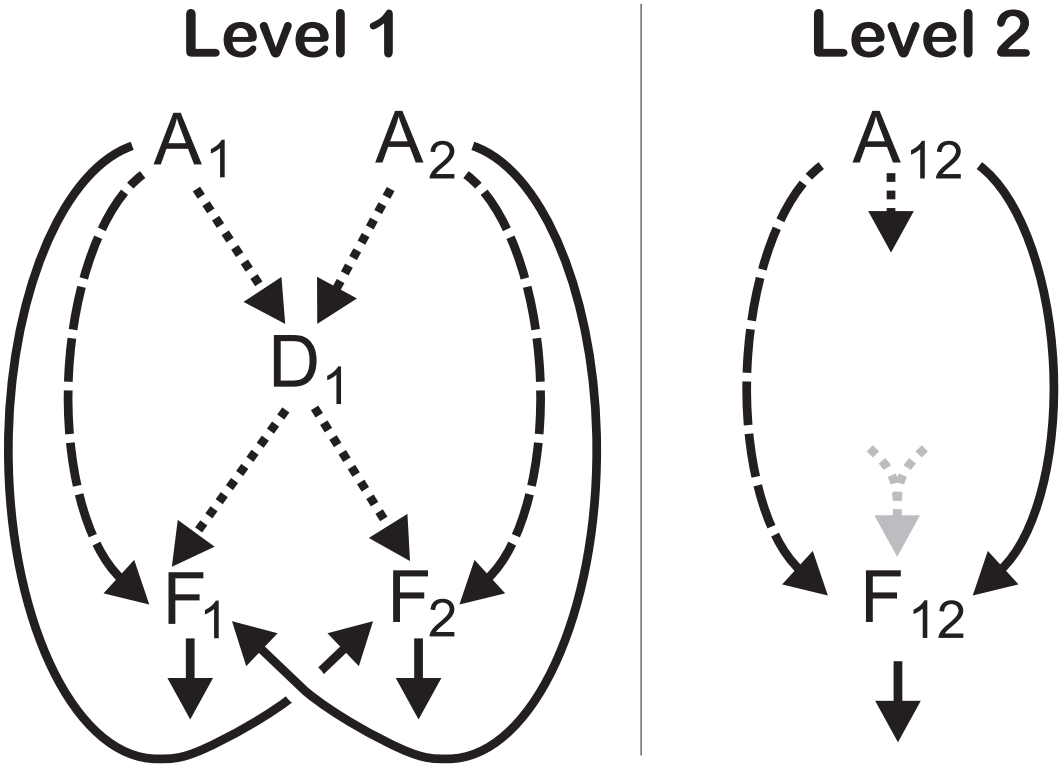

Figure 8: Reduced 1- and 2-cumomer networks from Figure 7.