An optimized algorithm for flux estimation from isotopomer distribution in glucose metabolites

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ABSTRACT

Motivation: Analysis of the conversion of 13C glucose within the metabolic network allows the evaluation of the biochemical fluxes in interconnecting metabolic pathways. Such analyses require solving hundreds of equations with respect to individual isotopomer concentrations, and this assumes applying special software even for constructing the equations. The algorithm, proposed by others could be improved.

Method: A C-code linked to the program written in Mathematica (Wolfram Research Inc.), constructs and solves differential equations for all isotopomer concentrations, using the general enzyme characteristics (\(K_m\), equilibrium constant, etc.). This code uses innovative algorithm of determination for the isotopomers–products, thus essentially decreasing the computation time. Feasible metabolic fluxes are provided by the parameters of enzyme kinetics found from the data fitting.

Results: The software effectively evaluates metabolic fluxes based on the measured isotopomer distribution, as was illustrated by the analysis of glycolysis and pentose phosphate cycle. The mechanism of transketolase and transaldolase catalysis was shown to induce a specific kind of isotopomer re-distribution, which, despite the significance of its effect, usually is not taken into account.

Availability: The software could be freely downloaded from the site: http://bq.ub.es/bioqint/label_distribution/

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INTRODUCTION

Quantification of metabolic fluxes located at the crossroads of several metabolic pathways is of high interest in physiological research. In particular, this refers to metabolism of glucose 6-phosphate (G6P), which can be directed towards synthesis of glycogen or metabolized through glycolysis (reactions 1, 6, 7, 8 and 10 of Fig. 1) or the pentose phosphate pathway (reactions 2, 3, 4, 5 and 9 of Fig. 1). Like the Krebs cycle, the pentose cycle performs both anaplerotic (for recycling of hexoses, pentoses and trioses) and cataplerotic (for NADPH production with loss of glucose carbon) functions. Two branches can be considered in this cycle: oxidative (reaction 2) and non-oxidative (reactions 3, 4, 5 and 9). The pentose phosphate cycle (PPC) is essential for the synthesis of RNA and DNA, and NADPH mainly for the synthesis of fatty acids in animal tissues, and increased fluxes through the PPC is a characteristic of proliferating cancer cells. The PPC can be subjected to increased fluxes in the case of carcinogenic transformation requiring a major rate of synthesis of pentoses and NADPH to cope with increased need of RNA, DNA and lipids.

Metabolism of 13C-labeled glucose within the metabolic network results in re-distribution of labeled atoms among the intermediates. Such distribution in the metabolites of PPC or glycolysis measured with nuclear magnetic resonance (NMR) or gas chromatography and mass spectrometry (GC–MS) can be used for flux estimations within this network. The first approach to such estimation suggested by Katz and Wood (1960) based on solving balance equations, describing isotopic steady state, with respect to fluxes and fractional labeling.

A typical problem of metabolic flux analysis using such metabolite balances is undetermined equation systems. Measuring fractional labeling often can provide unambiguous solution only suggesting unidirectionality of the fluxes, which cannot be correct in the view of reversibility of transketolase (TK) and transaldolase (TA) reactions. Accounting bidirectionality of the reactions increases the number of variables...
Recent advances in experimental techniques have made available not only the fractional enrichment in the individual carbon atom positions, but also mass isotopomer or even individual isotopomer distribution. Analysis of these experimental data demands simplifying suggestions (Katz and Rognstad, 1967) or additional experimental information (Follstad and Stephanopoulos, 1998; Wiechert and de Graaf, 1996). Using the commonly accepted biochemical parameters of enzymatic reactions ($K_m$, equilibrium constants, inhibitory constants, etc.) together with label distribution, as suggested here, could provide additional information for determination of the parameters.

Recent advance in experimental techniques have made available not only the fractional enrichment in the individual carbon atom positions, but also mass isotopomer or even individual isotopomer distribution. Analysis of these experimental data demands simplifying suggestions (Katz and Rognstad, 1967) or additional experimental information (Follstad and Stephanopoulos, 1998; Wiechert and de Graaf, 1996). Using the commonly accepted biochemical parameters of enzymatic reactions ($K_m$, equilibrium constants, inhibitory constants, etc.) together with label distribution, as suggested here, could provide additional information for determination of the parameters.

Advantages compared with the one suggested previously. The matrix equations (Schmidt et al., 1997) are an elegant form of presentation of hundreds of equations for isotopomers, however, they do not reduce the real number of calculations, which became larger than if the equations were written explicitly, and the calculation time could be a limiting factor for the analysis. At the same time, as most elements of isotopomer mapping matrix are zeros, the calculation process could be rationalized. Our functions, written in C for each enzyme of the considered metabolic pathway, have the same advantage of automatically constructed equations, but special organization of finding isotopomer–product for each isotopomer–substrate allows performing actually less calculations than the standard matrix multiplication suggested by Schmidt et al. (1997), essentially decreasing the computation time that in turn allows variability in choosing the time step necessary for the numerical solution of stiff systems.

In addition to the classical reactions catalyzed by the TK (F6P + G3P ↔ E4P + X5P; X5P + R5P ↔ S7P + G3P) and the TA (F6P + E4P ↔ S7P + G3P) other isotope exchange reactions were considered to account for the label distribution among the PPC components (Figs 1 and 2). As schematized in Figure 2, three of these reactions are related to the TK (X5P + G3P; S7P + R5P; F6P + E4P) and two of them to TA activity (S7P + E4P; F6P + G3P). Only two (not four) metabolites participate in them, as products are the same as the substrates. Though, not yielding new products, these reactions however redistribute the labeled atoms among the intermediates of PPC (Fig. 2). According to the commonly accepted reaction mechanism, for instance, after X5P binding to the TK, a moiety of two carbon atoms remains fixed to the active site and GAP is liberated. Such splitting of X5P is reversible and the reverse reaction normally restores the initial state. However, if differently labeled GAP molecules are present, the reverse reaction could result in differently labeled X5P. These reactions, since they do not change overall concentrations, referred below as ‘invisible’.

The solution of the system of differential equations gives the distribution of all possible isotopomers, which could be re-calculated to the mass isotopomers or percentage of label enrichment in a certain position. All these data could be used for the fit of corresponding experimental data providing feasible solutions of metabolic fluxes. Thus, wide range of the experimental data might be analyzed using the present software.

**SYSTEMS AND METHODS**

The software for analysis of isotopomer distribution in the metabolites of glycolytic and pentose phosphate pathways consists of two parts. The main program, written in Mathematica (Wolfram Research, Inc.), implements enzyme kinetic parameters for calculating the time course and steady state...
of overall concentrations of metabolites. Using the function 'SS', it solves the respective differential equations using any of the methods provided by Mathematica 5.0 (as implicitbackward differentiation formulas of orders 1 through 5, predictor–corrector Adams method of orders 1 through 12, etc.). This algorithm, which could be used independently for analysis of global metabolic fluxes and concentrations, is connected through the common parameters with the second part, written in C and connected to the Mathematica program using MathLink. It computes and handles the distribution of all isotopomers (in the presented case, produced from 1,2<sup>13</sup>C-glucose) formed in the reactions of PPC and glycolysis.

The label distribution algorithm formulates and solves differential equations for all isotopomers with the Euler method using the values of global fluxes obtained in the main Mathematica program. The solution, presented as sum of the obtained isotopomers concentrations, could be checked in each simulation by agreement with the overall metabolite concentration, obtained by the main (Mathematica) program.

Keeping the main program in the friendly Mathematica shell makes it open for modifications, while the linked C algorithm allows the execution of the bulk of calculations faster than in Mathematica.

The isotopomer distribution obtained using the function ‘label’ can be saved in separate data file with the function ‘savres’; the function ‘rdist’ reads these data file for recompilation. Two other functions, ‘rib’ and ‘lac’, calculate the mass isotopomer distribution in ribose and lactate for comparison with experimental values.

These functions are also used for fitting of mass isotopomer distribution as parts of the composite functions ‘sensitivity’, ‘error’, ‘jump’ and ‘descent’. Analogous functions ‘coner’, ‘consens’ and ‘condesc’ are used for fitting the overall metabolite concentrations.

The software is executable for Windows; on request we can provide a version for Linux, or make the complete program in C for Windows and/or Linux.

ALGORITHM

Analysis of the overall concentrations and fluxes

Function ‘SS’. The function ‘SS’, written in Mathematica, solves the system of differential equations (written here in Mathematica notation) describing interconversion of metabolites shown in Figure 1, not considering their isotopomers:

\[
\begin{align*}
S7P'[t] &= (V_3*NV[3] - V_5*NV[5])/Vol, \\
E4P'[t] &= (-(V_4*NV[4]) + V_5*NV[5])/Vol, \\
\end{align*}
\]

Here \(V_i\) are the reaction rates, where the index \(i\), varying from 1 to 10, corresponds to the numeration of the reactions in Figure 1. The reaction rates are functions of substrate concentrations accounting for the reaction mechanism, bidirectionality of the fluxes and the enzyme kinetics parameters (equilibrium constant, maximal velocities, regulatory properties and \(K_m\) and \(K_i\) values), which are summarized in Tables 1–3. For bisubstrate reactions, the \(K_m\) for one of the substrates is usually measured experimentally at non-physiologically high concentration of the other substrate. The apparent \(K_m\) could be different at lower second substrate concentration, or in the presence of products or competitive inhibitors. Moreover, not for all considered substrates the \(K_m\)
phosphate isomerase) are considered to be in fast equilibrium. Accordingly, the compounds H6P (F6P and G6-P), P5P (Rl5P, X5P and R5P) and T3P (GAP and DHAP), are considered as single pools, where the individual substances are distributed according to their equilibrium constant. Other variables are S7P, E4P and FBP. Only V\text{max} of non-equilibrium reactions are considered here as unknown parameters, which could be adjusted by fitting the experimental data; the fluxes are obtained automatically based on the known equilibrium constants and/or the rate equations. For convenience of the fitting procedure, the factors, which modify the individual reaction rates in the system of differential equations, are presented as competition between substrates. The array could be expanded according to specific needs, so that other parameters could be modified. Vol in the system (1) is the intracellular volume corresponding to 1 mg of cellular protein.

Solving the differential equations the software provides the evolution of system toward steady state. If the metabolite concentrations are determined experimentally, they could be

Table 2. The used in the model values of equilibrium constants of the reactions in the analyzed pathway

<table>
<thead>
<tr>
<th>Enzyme and mass ratio</th>
<th>K\text{eq}</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>EP&amp;RPL (X5P/R5P)</td>
<td>1.5</td>
<td>Casazza and Veech (1986)</td>
</tr>
<tr>
<td>TK: X5P × R5P/(Gra3P × S7P)</td>
<td>0.48</td>
<td>Casazza and Veech (1986)</td>
</tr>
<tr>
<td>Gra3P × F6P/E4P × X5P</td>
<td>29.7</td>
<td></td>
</tr>
<tr>
<td>TA: E4P × F6P/(Gra3P × S7P)</td>
<td>0.37</td>
<td>Casazza and Veech (1986)</td>
</tr>
<tr>
<td>PGI: F6P/G6P</td>
<td>0.28</td>
<td>Benevolensky et al. (1994)</td>
</tr>
<tr>
<td>Aldolase (mM)</td>
<td>0.086</td>
<td>Connell (1985) and Cornell et al. (1979)</td>
</tr>
<tr>
<td>Gra3P × DHAP/FBP</td>
<td>0.047</td>
<td>Veech et al. (1969)</td>
</tr>
</tbody>
</table>

Table 3. Michaelis constants of the reactions in analyzed pathway

<table>
<thead>
<tr>
<th>Enzyme and substrate</th>
<th>K\text{m} (mM)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>TK</td>
<td>0.013</td>
<td>Heinrich et al. (1976)</td>
</tr>
<tr>
<td>S7P</td>
<td>0.006</td>
<td>Heinrich et al. (1976)</td>
</tr>
<tr>
<td>R5P</td>
<td>0.02</td>
<td>Heinrich et al. (1976)</td>
</tr>
<tr>
<td>GAP</td>
<td>0.74</td>
<td></td>
</tr>
<tr>
<td>F6P</td>
<td>0.7</td>
<td>Sprenger et al. (1995)</td>
</tr>
<tr>
<td>E4P</td>
<td>0.0004</td>
<td>Sprenger et al. (1995)</td>
</tr>
<tr>
<td>TA</td>
<td>0.003</td>
<td>Heinrich et al. (1976)</td>
</tr>
<tr>
<td>F6P</td>
<td>0.0004</td>
<td>Heinrich et al. (1976)</td>
</tr>
<tr>
<td>GAP</td>
<td>0.04</td>
<td>Heinrich et al. (1976)</td>
</tr>
<tr>
<td>S7P</td>
<td>0.036</td>
<td></td>
</tr>
<tr>
<td>FPK</td>
<td>F6P</td>
<td>0.5</td>
</tr>
<tr>
<td>Ald</td>
<td>FBP</td>
<td>0.01</td>
</tr>
<tr>
<td>GAP</td>
<td>DHAP</td>
<td>0.016</td>
</tr>
<tr>
<td>FBPase</td>
<td>FBP</td>
<td>0.029</td>
</tr>
<tr>
<td>G6PDH</td>
<td>0.1</td>
<td></td>
</tr>
</tbody>
</table>

RPI, ribose-5-phosphate isomerase (E.C. 5.3.1.6); EP, epimerase (E.C. 5.1.3.1); PGI, glucose phosphate isomerase (E.C. 5.3.1.9); TPI, triose phosphate isomerase (E.C. 5.3.1.1); TK, transketolase (E.C. 2.2.1.1); TA, transaldolase (E.C. 2.2.1.2); PFK, phosphofructokinase (E.C. 2.7.1.11); aldolase (E.C. 4.1.2.13); F1,6Pase, fructose 1,6 bisphosphatase (E.C. 3.1.3.11).

Table 1. Kinetic mechanism of the enzymatic reactions in analyzed pathway

<table>
<thead>
<tr>
<th>Enzyme and Catalyzed reaction</th>
<th>Reaction mechanism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidative branch G6P → R15P</td>
<td>Michaelis–Menten</td>
<td>Fersht (1977)</td>
</tr>
<tr>
<td>EP R15P ↔ Xyl15P</td>
<td>Fast equilibrium</td>
<td>Casazza and Veech (1986)</td>
</tr>
<tr>
<td>RPI Xyl15P ↔ R5P</td>
<td>Fast equilibrium</td>
<td>Casazza and Veech (1986)</td>
</tr>
<tr>
<td>TK Xyl15P + R5P ↔ S7P + Gra3P</td>
<td>Ping-pong</td>
<td>McIntyre et al. (1989)</td>
</tr>
<tr>
<td>TK E4P + Xyl15P ↔ Gra3P + F6P</td>
<td>Ping-pong</td>
<td>McIntyre et al. (1989)</td>
</tr>
<tr>
<td>TA S7P + Gra3P ↔ E4P + F6P</td>
<td>Ping-pong</td>
<td>McIntyre et al. (1989)</td>
</tr>
<tr>
<td>PGI F6P ↔ G6P</td>
<td>Fast equilibrium</td>
<td>Casazza and Veech (1986)</td>
</tr>
<tr>
<td>PFK F6P + ATP ↔ FBP + ADP</td>
<td>Sequential</td>
<td>Brand and Soling (1974)</td>
</tr>
<tr>
<td>F1,6Pase F1,6P → F6P + Pi</td>
<td>Ordered bi–bi</td>
<td>Stone and Fromm (1980)</td>
</tr>
<tr>
<td>Aldolase Gra3P + DHAP ↔ FBP</td>
<td>Ordered bi–uni</td>
<td>Tsolas and Horecker (1972)</td>
</tr>
<tr>
<td>TPI Gra3P + DHAP</td>
<td>Fast equilibrium</td>
<td>Casazza and Veech (1986)</td>
</tr>
</tbody>
</table>
compared with calculation and used for adjusting the parameters in the array NV. The global fluxes, obtained at this step, are used as parameters for the second step of analysis, calculating percentage of all isotopomers of the compounds presented in Figure 1.

**Analysis of isotopomer concentrations**

*Function ‘label’* The second step of the algorithm with the central function ‘label’, written in C and linked via MathLink tools to the main program, constructs and solves the system of differential equations for all isotopomers of all metabolites presented in Figure 1 using the values of global fluxes obtained by the function ‘SS’ of the main program.

A binary notation for the $^{13}$C and $^{12}$C atoms, similar to that in Schmidt et al. (1997), was used for isotopomer designation. Since each carbon atom of a molecule can be in one of the two states: labeled (marked as ‘1’) or unlabeled (‘0’), each metabolite in the model can be represented by an array of $2^n$ possible isotopomers, where $n$ is the number of carbon atoms in a molecule. Each isotopomer in the model is presented as a string of binary digits, whose length corresponds to the number of carbon atoms in respective molecule (3 digits for trioses, 4 for erythrose, etc.). ‘1’ or ‘0’ in certain position in a string signifies that corresponding carbon atom is labeled or unlabeled. For instance, all isotopomers for GAP are presented in the following array:

$$000, 001, 010, 011, 100, 101, 110, 111$$ (2)

In this way, the isotopomers could be recognized as binary integers pointing to the position of the respective isotopomer in the array. This ordering of the isotopomers in the array allows optimization of finding the isotopomer products for any isotopomer substrate of the considered reactions, as is explained below.

The concentrations of the isotopomers are presented by real numbers arranged in the same order, so that the number of an isotopomer points to the position of its concentration. For instance, the value of concentration of an isotopomer of GAP ‘101’, representing the binary equivalent of 5, can be found as an element 5 in the array of concentrations for GAP isotopomers.

Special functions were designed for simulation of each kind of biochemical reactions between isotopomers. For instance, the function simulating TK reaction, according to its mechanism simulates cleavage of the C–C bond between the second and third carbon atoms of substrate–donor decreasing the value of respective donor concentration and simultaneously increasing the concentration corresponding to the released acceptor–product. Binding the remaining two-carbon part with the existed substrate–acceptor is simulated as decrease in the related substrate–acceptor concentration and increase in the concentration of the donor–product, whose reference number presented as concatenated two first digits of the donor–substrate with the number of acceptor–substrate. For instance, the reaction (X5P + R5P $\leftrightarrow$ S7P + GAP) between arbitrarily chosen isotopomers of X5P (10011) and R5P (10000) produces the following isotopomers of S7P and GAP:

$$10\,011 + 10\,000 \leftrightarrow 10\,10\,000 + 011$$ (3)

In this example, the X5P isotopomer is presented as binary code of integer 19 and its reference number is 19. The product of its splitting (GAP, 011) analogously has the reference number 3. Note that $3 = 19 - 2 \times 8$, $8$ is the number of all triose isotopomers, and 2 is the integer part of 19/8. The same isotopomer of GAP will appear after splitting of any of X5P isotopomers with reference number $3 + k \times 8$, where $k = 0, 1, 2, 3$. Splitting of any X5P isotopomer number $i$ will give GAP with the reference number $i - 8 \times \left\lfloor \frac{i}{2(8-2)} \right\rfloor$.

The new donor in (3) (S7P, 101000) has the reference number 80, which is composed of $32 \times 16$: 32 is the number of pentose isotopomers, 16 is the reference number of chosen R5P isotopomer in (3), and 2 is the binary code of the X5P part after its splitting (10–011). Easy to check that for any X5P number $i$ and R5P number $j$, the produced S7P will have the reference number $32 \times \left\lfloor \frac{i}{8} + j \right\rfloor$. For any donor–substrate number $i$ with $n$ carbons and acceptor–substrate number $j$ with $m$ carbons, the resulting donor molecule will have the reference number $\left\lfloor j + 2^m \times \left\lfloor \frac{i}{2(8-2)} \right\rfloor \right\rfloor$.

Thus, the program, taking one by one the isotopomers of donor $i$ with $n$ carbons and acceptor $j$ with $m$ carbons determines the reference numbers of resulting acceptor

$$ra = \left\lfloor i - 2(n-2) \times \left\lfloor \frac{i}{2(8-2)} \right\rfloor \right\rfloor$$

of $n - 2$ carbons and donor

$$rd = \left\lfloor j + 2^m \times \left\lfloor \frac{i}{2(8-2)} \right\rfloor \right\rfloor$$

of $m + 2$ carbons.

Determining the reference numbers of products for each pair of substrates is the basis of the optimization. Then the concentrations must be recalculated according to the succession of reaction. Assuming that all isotopomers have the equal affinity, the rate of reaction between pair of isotopomers is proportional to their concentrations, while sum of the reaction rates for all isotopomers is to give the global metabolic flux calculated by the function ‘SS’. If, for instance, $V3f$ is the global forward flux for the TK reaction between X5P and R5P (defined using the function ‘SS’), the flux for this reaction between isotopomers $i$ and $j$ ($V3fi_j$) would be expressed as follows:

$$V3fi_j = V3f \times [X5P_i] \times [R5P_j] / ([X5P_{tot}] \times [R5P_{tot}]).$$ (4)

Here, the indices $i$ and $j$ refer to the concentration of the respective isotopomers and the index ‘tot’ refers to the overall
concentration of the metabolite, as calculated by the ‘SS’. This
is the way of connection of the first algorithm with the second
one, calculating the label distribution: the function ‘SS’ cal-
culates global fluxes and concentrations and defines the values
[as in the above example \(V3f/[X5P_{tot} \times [R5P_{tot}])\), which
are used by the function ‘label’ for calculations of the fluxes in
reactions between isotopomers [as in the Equation (4)]. This
gives the possibility of checking the consistency of fluxes in
the network and avoiding unreal steady states even before the
isotopomer analysis.

During the small time interval \(dt\) the considered as
an example TK reaction (3) consumes the amount \(dt \times V3f_{ij}\)
of each of the isotopomers \(i\) and \(j\) and this value is subtracted
from the concentrations of respective isotopomers.

\[
[X5P]_{t+dt} = [X5P]_t - dt \times V3f_{ij}
\]
\[
[R5P]_{t+dt} = [R5P]_t - dt \times V3f_{ij}
\]

Here, the indices \(t\) and \(t + dt\) indicate the time of the simu-
lated process. As usual for numerical solution of differential
equations, this could be acceptable approximation if the time
step \(dt\) is so small that consumption during one step is small
compared to the amount of these isotopomers; practically, the
value for \(dt\) is taken so that its further decrease does not affect
the solution.

The program adds the same amount to the concentrations
of reaction products, which numbers \((ra\) and \(rd\)\) are defined
from \(i\) and \(j\) as described above.

\[
[GAP]_{t+dt} = [GAP]_t + dt \times V3f_{ij}
\]
\[
[S7P]_{t+dt} = [S7P]_t + dt \times V3f_{ij}
\]

Thus, the present approach performs four multiplications
and summations for recalculating the concentrations of four
participating isotopomers. In contrast, calculation of trans-
formation of one isotopomer \(X5P\) to one isotopomer \(S7P\) in
the above reaction using the approach proposed earlier (Schmidt
et al., 1997) would demand multiplication of one row of 32
members of the isotopomer mapping matrix by 32 members
of \(X5P\) isotopomer vector and subsequent summation of the
products. Most of the multiplication products would be zeros.
Thus, our method performs fewer calculations and therefore
works faster. In fact such optimization of our software reduced
the calculation time by an order of magnitude. Moreover,
calculation of the total steady-state concentrations using the
function ‘SS’ allows for taking them as initial conditions for
subsequent isotopomer analysis, which in this case will only
change the distribution but not the overall concentration. This
could also decrease the calculation time in the case of stiff systems.

‘Invisible’ reactions were simulated analogously with an
exception that substrates are the same substances as products
but different isotopomers, for instance:

\[
10\, 100\times (X5P) + 000\times (GAP) \leftrightarrow 10\, 000\times (X5P) + 100\times (GAP).
\]

Transaldolase reactions were simulated with the same
function with exception that three carbons instead of two
remain bound with the enzyme after the donor splitting.

For monosubstrate reactions the algorithm is much simpler:

\[
\text{G6PDH: } 110\, 000 \rightarrow 10\, 000;
\]
\[
\text{Aldolase reaction: } 110\, 100 \leftrightarrow 011 + 100;
\]

Here, the order of atoms in the first triose is reverse comparing
to that in hexose in accord with the reaction mechanism.

For the forward direction of glycolysis, phosphofructo-
kine (PFK) reaction, which does not produce new hexose
isotopomers, the reaction is considered in conjunction with
the forward aldolase reaction, and for the reverse direc-
tion, the fructose 1,6-bisphosphatase reaction is considered
together with reverse aldolase reaction. Isomerization steps
(glucose phosphate isomerase, triosephosphate isomerase,
epimerase, ribosephosphate isomerase) are considered in fast
equilibrium and the ratio for each pair of corresponding iso-
topomers is calculated according to equilibrium constant for the
isomers.

In this way, the algorithm actually formulates and solves
the differential equations describing evolution of all the
isotopomers toward steady state.

Since the functions ‘SS’ and ‘label’ use the same values of
parameters, the steady states obtained by both functions must
be equivalent, i.e. sum of isotopomer concentration obtained
using ‘label’ must be equal to the respective concentration
of a substance obtained using ‘SS’. This provides a way of
checking the accuracy of isotopomer calculation by compari-
on of the steady states obtained by both functions. If there
were an error in computation, the values of overall concen-
trations, obtained by these two ways, would be different. An
error could result, for instance, from too big time step \(dt\), and
in case of inconsistency it must be reduced.

Ancillary functions

Function ‘savres’ saves the results obtained by the function
‘label’ writing three files in the working directory. In ‘distr.dat’
the calculated concentrations of all isotopomers are grouped
in a format, which allow (after simple editing procedure) its
inclusion as initial conditions in the C file after recompila-
tion of the C-program. Each subsequent first execution of
the function ‘label’ will start with those initial values. In the
file ‘discon.dat’ the same information is saved, but using the
format, which could be read using the function ‘rdist’, thus
switching to the desirable initial values without recompilation
of the program.

In the third file, ‘ribose.dat’, the time course of the concen-
tration of non-labeled isotopomer of R5P is written. It could
be used for visual control of steady state in isotopomers.
some experimental techniques allow determination of mass isotopomers (molecules with specific number of labeled atoms regardless of position of label). In this case, for comparison with the experimental data, the percentage of mass isotopomers must be computed from the obtained array of all isotopomers. Such recalculations are at present available for pentoses (function ‘rib’) and trioses (function ‘lac’). These functions first calculate the overall concentrations of R5P and GAP, respectively, as a sum of all isotopomer concentrations. The agreement of these values with the corresponding values obtained by the function ‘SS’ can be verified. Then concentrations of the molecules with certain fixed number of labeled atoms, and their ratio to the overall concentration is evaluated. Functions ‘rib’ and ‘lac’ return an array containing the percentage of the mass isotopomers with 0 – (n – 1) labels and the overall concentration of the mass isotopomer. Similar functions can be easily constructed for calculation of mass isotopomers of other compounds.

**IMPLEMENTATION AND DISCUSSION**

**Checking the metabolic steady state**

Since the differential equations are solved numerically, checking the achievement of metabolic steady state within the chosen time interval is necessary. Figure 3A shows the graphic output for R5P concentrations obtained by the function ‘SS’, indicating that steady state is practically achieved after 0.4 min. The steady state for isotopomers, calculated using the function ‘label’ could be verified in a similar way. Figure 3B shows the time course of unlabeled isotopomer of R5P calculated with the same values of parameters, plotted from the file ‘ribose.dat’ saved automatically after execution of the function ‘savres’. If the flux through oxidative branch of PPC is set to zero (NV[[2]] = 0) as well as TA reaction (NV[[5]] = NV[[14]] = NV[[15]] = 0), only few isotopomers could be formed from 100% [1,2\(^{13}\)C\(_2\)glucose. Their formation could be predicted as shown in Figure 4 and respective simulation (started from zero initial concentration for all isotopomers) agrees with this prediction.

**Construction of isotopomers**

In the complete metabolic system shown in Figures 1 and 2, all possible isotopomers could be formed, and this makes difficult checking the performance of program. In reduced system, where the flux through oxidative branch of PPC is set to zero (NV[[2]] = 0) as well as TA reaction (NV[[5]] = NV[[14]] = NV[[15]] = 0), only few isotopomers could be formed from 100% [1,2\(^{13}\)C\(_2\)glucose. Their formation could be predicted as shown in Figure 4 and respective simulation (started from zero initial concentration for all isotopomers) agrees with this prediction.

**Fitting the experimental data**

Functions ‘error’, ‘sensitivity’, ‘jump’, ‘descent’ are designed for fitting procedure minimizing the square of difference between the computed and experimental values. They

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**Table 4.** Overall R5P and GAP concentrations obtained by solving the system of differential equations corresponding to the scheme in Figure 1 (function ‘SS’) and as the sum of isotopomers (function ‘label’), obtained using the following set of parameters: \( NV = \{ 1., 0.89, 0.45, 0.79, 0.855, 0.483, 1., 0.125, 1., 1.22, 2, 0.5, 0.955, 1, 32.9 \} \)

<table>
<thead>
<tr>
<th>Tool</th>
<th>R5P</th>
<th>GAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘SS’</td>
<td>0.03997</td>
<td>0.024097</td>
</tr>
<tr>
<td>‘label’</td>
<td>0.04061</td>
<td>0.024077</td>
</tr>
</tbody>
</table>

---

**Fig. 3.** The time course of overall R5P concentration obtained with the function ‘SS’ (A) or non-labeled isotopomer of R5P in the first (B) and second (C) run of the function ‘label’ with time step \( dt = 0.00001 \) min; and the following set of other parameters: \( NV = \{ 1., 0.89, 0.45, 0.79, 0.855, 0.483, 1., 0.125, 1., 1.22, 2, 0.5, 0.955, 1, 32.9 \} \)
Fig. 4. All possible isotopomers predicted for the reduced system, without oxidative branch of PPC and TA reaction \((\text{NV}[2] = 0 \text{ and } \text{NV}[5] = \text{NV}[14] = \text{NV}[15] = 0)\). Splitting of initial hexose (I) in reaction 7 (Fig. 1) results in two different isotopomers of trioses, which recombination in the backward reaction, taking into account label interchange in the triose isomerase reaction, gives four different isotopomers of hexose. TK reaction (4) between all possible couple triose and hexose isotopomers gives two possible isotopomers of erytrose and four isotopomers of pentose, which are equally distributed in R5P and X5P due to isomerase reaction. Interaction of the two pentoses in TK reaction (3) gives eight isotopomers of S7P and does not give any new isotopomers of trioses.

could be combined and modified according to the specific experimental data. An example of the fitting procedure is included in the Mathematica program. It assumes that overall metabolite concentration, and mass isotopomer percentages of trioses and pentoses are defined experimentally and represent the objective of fitting.

Before starting the fitting procedure, the function ‘error’ finds the initial difference between experimental and simulation data at an arbitrarily chosen set of parameters. This function executes the described above functions ‘SS’, ‘label’, then ‘rib’ and ‘lac’, and finally finds sum of square difference between the calculated and measured mass isotopomer distribution, separately for R5P and GAP.

Then sensitivity of the error to every parameter, designed for fitting, is checked using the function ‘sensitivity’. The array nNV points to the set of parameters designed for fitting. Each element in nNV enumerates the position of a parameter in the array NV. For instance, if the rates of reactions 2, 5, 6, 7, 10, 11 are designed for fitting, the array nNV consists of these numbers. Function ‘sensitivity’ changes the chosen parameters one by one, taking in the presented case 0.9 of its value, executes ‘error’, finds the difference between the new and initial error, and before taking the next parameter, restores the value of the previous one. After performing all the demanded parameter changes, it finds relative sensitivity to all parameters, the maximal error change, its sign and the parameter of maximal sensitivity, to which the maximal error change refers.

The parameter of maximal sensitivity could be changed for better fitting using the function ‘jump’. Alternatively, all of the parameters can be changed proportionally using the function ‘descent’. These two ways or modifying initial parameters for fitting could be combined, as realized in the present version.

After the change of parameters, the resulting difference between calculated and experimental data is compared with the initial one and in case if it is decreased, the new set of parameters could be taken and the procedure of fitting starts again. Since the calculation of overall concentrations takes much less time than that for label distribution, and the overall concentration could be an object of fitting, the functions ‘coner’, ‘consens’, ‘condesc’ were developed, which act analogously to the described ones, but designed only for fitting the overall concentrations, without execution of the function ‘label’.

The isotopomer distributions resulting from the fitting procedure starting from an arbitrarily chosen array of parameters are shown in Table 5 under ‘Fit 1’. The set of parameters obtained from fitting the mass isotopomer distribution defines corresponding global fluxes shown in Table 6. Since the experimental distribution of R5P and lactate isotopomers actually are not sufficient for unambiguous determination of fluxes in the considered network, different sets of parameters could result in similar isotopomer distribution. In this case if more experimental data designed for fitting were available, it would restrict the acceptable range of parameters. In the present example, after obtaining the set ‘Fit 1’, adding the overall concentrations shown in Table 7 expands the set of experimental data designed for fitting. In this case, the fitting procedure can be continued leading to the parameter set named ‘Fit 2’. As is shown in Table 5 ‘Fit 2’ gives the mass isotopomer distribution similar to the ‘Fit 1’, but the different corresponding fluxes, shown in Table 6, thus demonstrating insufficiency of the mass isotopomer data for restricting the parameter set. In this case, total concentration could serve as the restricting factor. As Table 7 shows, ‘Fit 2’ gives concentrations, which are more close to the goal values than those corresponding to the ‘Fit 1’. Owing to the lack of experimental data, in this particular example the isotopomer distribution (our experiment on HT29 cells) and the overall concentrations (Casazza and Veech, 1986) were taken.
Table 5. Available steady state mass isotopomer distribution in H29 cells growing in the medium containing 100% 1,2-C$^{13}$-glucose and the corresponding computed distribution obtained with the same set of parameters as for the Table 4 (Fit 1), change of the distribution after substituting the factors for ‘invisible’ reactions (last five parameters) by 0.001 and with the set of parameters [Fit 2: 1, 0.42, 0.6, 0.93, 1.49, 0.45, 1.06, 0.13, 1, 7, 2.66, 5.72, 0.16, 5.53, 12.5] obtained after subsequent simultaneous fitting of the label distribution and overall concentrations presented in Table 7.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Measured</th>
<th>Fit 1</th>
<th>Fit 1 without ‘invisible’</th>
<th>Fit 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>R5P</td>
<td>0.165</td>
<td>0.152</td>
<td>0.044</td>
<td>0.16</td>
</tr>
<tr>
<td>1</td>
<td>0.45</td>
<td>0.47</td>
<td>0.816</td>
<td>0.47</td>
</tr>
<tr>
<td>2</td>
<td>0.098</td>
<td>0.126</td>
<td>0.045</td>
<td>0.11</td>
</tr>
<tr>
<td>3</td>
<td>0.224</td>
<td>0.21</td>
<td>0.061</td>
<td>0.227</td>
</tr>
<tr>
<td>4</td>
<td>0.068</td>
<td>0.04</td>
<td>0.033</td>
<td>0.028</td>
</tr>
<tr>
<td>Triose</td>
<td>0.524</td>
<td>0.52</td>
<td>0.513</td>
<td>0.51</td>
</tr>
<tr>
<td>1</td>
<td>0.021</td>
<td>0.027</td>
<td>0.023</td>
<td>0.014</td>
</tr>
<tr>
<td>2</td>
<td>0.452</td>
<td>0.43</td>
<td>0.461</td>
<td>0.46</td>
</tr>
</tbody>
</table>

The fluxes are expressed in µM/min/mg of protein.

Table 6. Fluxes calculated for two different sets of parameters, same as those used for the Table 5.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Fit 1</th>
<th>Fit 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>2: oxidative PPC, G6P → R5P</td>
<td>0.016</td>
<td>0.0078</td>
</tr>
<tr>
<td>14: TA, F6P + GAP</td>
<td>0.287</td>
<td>0.28</td>
</tr>
<tr>
<td>15: TA, S7P + E4P</td>
<td>1.05</td>
<td>0.7</td>
</tr>
<tr>
<td>5: TA, S7P + GAP ↔ E4P + F6P</td>
<td>0.022</td>
<td>0.0186</td>
</tr>
<tr>
<td>5: TA, S7P + GAP ↔ E4P + F6P</td>
<td>0.017</td>
<td>0.016</td>
</tr>
<tr>
<td>11: TK, X5P + GAP</td>
<td>0.00006</td>
<td>8.5 × 10^{-6}</td>
</tr>
<tr>
<td>13: TK, F6P + E4P</td>
<td>4.3 × 10^{-6}</td>
<td>3.9 × 10^{-6}</td>
</tr>
<tr>
<td>12: TK, S7P + R5P</td>
<td>0.012</td>
<td>0.0039</td>
</tr>
<tr>
<td>3: TK, R5P + X5P → GAP + S7P</td>
<td>0.0057</td>
<td>0.0027</td>
</tr>
<tr>
<td>3: TK, R5P + X5P ↔ GAP + S7P</td>
<td>0.00029</td>
<td>0.0002</td>
</tr>
<tr>
<td>4: TK, X5P + E4P → GAP + F6P</td>
<td>0.0072</td>
<td>0.0029</td>
</tr>
<tr>
<td>4: TK, X5P + E4P ↔ GAP + F6P</td>
<td>0.0018</td>
<td>0.0004</td>
</tr>
<tr>
<td>6: PFK, F6P → FBP</td>
<td>0.13</td>
<td>0.13</td>
</tr>
<tr>
<td>8: F6PASE, F6P ↔ FBP</td>
<td>2.1 × 10^{-6}</td>
<td>7.6 × 10^{-8}</td>
</tr>
<tr>
<td>9: R5P →</td>
<td>0.00025</td>
<td>0.0001</td>
</tr>
<tr>
<td>10: GAP →</td>
<td>0.266</td>
<td>0.27</td>
</tr>
</tbody>
</table>

The fluxes are expressed in µM/min/mg of protein.

Table 7. Overal concentrations measured by Casazza and Veech (1986) for rat liver and calculated for two different sets of parameters, same as those used for the Table 5.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Measured</th>
<th>Fit 1</th>
<th>Fit 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>R5P</td>
<td>0.006</td>
<td>0.034</td>
<td>0.019</td>
</tr>
<tr>
<td>S7P</td>
<td>0.05</td>
<td>0.006</td>
<td>0.019</td>
</tr>
<tr>
<td>GAP</td>
<td>0.001</td>
<td>0.024</td>
<td>0.0038</td>
</tr>
</tbody>
</table>

‘Fit 1’ is the parameter set obtained by fitting only mass isotopomer distribution presented in Table 5. ‘Fit 2’ is the parameter set obtained after subsequent simultaneous fitting of the label distribution and overall concentrations. The concentrations are expressed in mM. from different sources. However, such data combination could be used to demonstrate the restrictions caused by additional experimental data (total concentrations) for the parameter estimation. In addition, the suggested approach allows other kinetic measurements such as $V_{\text{max}}$, ratio between $V_{\text{max}}$ for different reactions performed by the same enzyme, rate constants for elementary steps, etc to be included in the analysis. Such complete information would provide evaluation of an unambiguous set of parameters and fluxes in the analyzed network. In the case of excessive information, the algorithm could be used for verification of the correspondence of the reaction mechanism and parameters obtained mainly in vitro with that defining the reactions in vivo.

Thus, the software could accumulate and use for the analysis of diverse known data regarding the enzyme activities in the considered network and the reaction mechanisms. Since these data differ between various organisms and tissues, the model must be organism- and tissue-specific and initial parameters given in the present demonstration version should be changed according to the specific task.

Table 5 shows the role of ‘invisible’ reactions in label distribution; calculation with zero ‘invisible’ flux and the same factors for other reactions as in ‘Fit 1’ gives essentially different mass isotopomer distribution. Thus, ignoring this kind of isotope exchange could result in the misinterpretation of experimental data. We use the term ‘invisible’ just for short reference to well-known TK and TA reactions, which in fact represent half of whole reaction cycle for the usually considered reactions. For instance, the following reversible steps represent the first half of the TK reaction X5P + R5P ↔ S7P + GAP: X5P binding, its catalytic splitting and the release of GAP. These steps are exactly what we call ‘invisible’ reaction (X5P + GAP). These steps are commonly accepted (see the scheme in Solov’eva et al., 2000) as a part of the whole reaction, but can take place even if only one substrate–donor (X5P) is present (Bykova et al., 2001). However, the contribution of these half-reaction in isotope exchange is usually not considered.

Such view makes evident that the same rate constants for elementary steps define the whole catalytic cycle as well as so called ‘invisible’ part of it. In this way, the values of whole and ‘invisible’ fluxes defined for a specific object could then be used for the evaluation of elementary rate constants—objective of particular interest for understanding the different aspects of enzyme functioning (competition between substrates, reversibility, etc.) in vitro in addition to the data obtained earlier in vitro (Selivanov et al., 1997, 2004).

CONCLUSIONS

A new approach is suggested for the analysis of GC–MS data aimed in evaluation of metabolic fluxes. It has the following
improvements over existing approaches.

(1) Based on the taken parameters of enzyme kinetics, it calculates the overall fluxes and steady-state concentrations of the metabolites, which then are used for the isotopomer analysis. This allows avoiding unreal steady states, decreasing calculation time and increasing accuracy in the isotopomer analysis. It restricts the acceptable range of parameters by fitting the overall concentrations in addition to the label distribution and using the known parameters of the considered enzymatic reactions.

(2) The part, which automatically formulates and solves the differential equations for the isotopomer concentrations, is optimized comparing to the analogous approach first described in Schmidt et al. (1997). A new algorithm of determination the isotopomer product for each isotopomer substrate reduces the number of calculations over that using the isotopomer mapping matrices. This optimization potentially reduces the calculation time an order of magnitude.

(3) The parts of transketolase and transaldolase catalytic cycle, effectively redistributing labeled carbon atoms within the metabolites without affecting their overall concentrations, are taken into account for the first time.

(4) The software, presented here in the general form, is dedicated for the analysis of glucose carbon distribution in the central metabolism, contains various specific information and therefore must be adapted to a given organism or type of cells.

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REFERENCES


