MINIREVIEW

13C Metabolic Flux Analysis

Wolfgang Wiechert

Department of Simulation, IMR, University of Siegen, Paul-Bonatz-Strasse 9-11, D-57068 Siegen, Germany

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Metabolic flux analysis using 13C-labeled substrates has become an important tool in metabolic engineering. It allows the detailed quantification of all intracellular fluxes in the central metabolism of a microorganism. The method has strongly evolved in recent years by the introduction of new experimental procedures, measurement techniques, and mathematical data evaluation methods. Many of these improvements require advanced skills in the application of nuclear magnetic resonance and mass spectrometry techniques on the one hand and computational and statistical experience on the other hand. This minireview summarizes these recent developments and sketches the major practical problems. An outlook to possible future developments concludes the text. © 2001 Academic Press

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INTRODUCTION

In recent years, metabolic flux analysis (MFA) has become one of the major tools in metabolic engineering. The aim of MFA is the detailed quantification of all metabolic fluxes in the central metabolism of a microorganism. The result is a flux map that shows the distribution of anabolic and catabolic fluxes over the metabolic network (Fig. 1). Based on such a flux map or a comparison of different flux maps, possible targets for genetic modifications might be identified, and the result of an already performed genetic manipulation can be judged or conclusions about the cellular energy metabolism can be drawn.

Stoichiometric MFA

The foundations of MFA were laid in the early 1990s with the stoichiometric MFA (Varma and Palsson, 1994). This method solely relies on the known stoichiometry of a given biochemical reaction network. The only necessary assumption is that the biological system is in a stationary or quasistationary state which means that all intracellular pool sizes do not change over the time span where the experiment takes place. This assumption certainly holds true in a continuous culture and with some care also in the exponential phase of a batch culture or in a batch-fed culture with slow variations in the input feed.

Under these conditions, the incoming fluxes of each intracellular metabolite pool balance the outgoing fluxes which gives rise to one linear equation for each intracellular pool. Figure 2a illustrates this concept for a very simple example network. In this network, the stationary condition assumption yields the following set of linear flux relations:

\[ u = q + v, \quad v = w, \quad w = p, \quad p + q = r + v. \]

These are four relations for a total of six flux variables which means that the space of all possible flux patterns of the network is constrained to a two-dimensional linear subspace of the total six-dimensional flux space. If at least two of the six fluxes can be directly measured, as for instance is always the case with substrate uptake, growth rate, product formation, by-product formation, or CO2 evolution, it might be possible to calculate all the fluxes from the stoichiometric relations and measurements. These measurable fluxes are called the extracellular fluxes. If in the example the extracellular fluxes \( u \) and \( r \) are measured, then all the other fluxes can be calculated from the stoichiometry as

\[ v = u - r, \quad w = u - r, \quad p = u - r, \quad q = r. \]

This procedure led to the first flux maps for the complete central metabolism in the early 1990s (Vallino and Stephanopoulos, 1993).

Shortcomings of Stoichiometric MFA

Unfortunately stoichiometric MFA is strongly limited and fails in the following situations:
1. In the case of parallel metabolic pathways (Fig. 3a) where none of the branches is coupled to a measurable variable, it is impossible to resolve the two branch fluxes. An example would be the two lysine-producing pathways in *Corynebacterium glutamicum* (Sonntag *et al.*, 1993).

2. Metabolic cycles which are not coupled to measurable fluxes cannot be resolved. Figure 3b shows an example in which a measurement of the in-flux is not sufficient to determine the fluxes in the metabolic cycle. For fixed extracellular fluxes the cycle flux can have an arbitrary value.

3. A special case of a metabolic cycle is a bidirectional reaction step which takes place in both directions at the same time (Fig. 3c). This situation is not an exception but rather the rule in a metabolic pathway because only those reactions that are strongly irreversible for thermodynamic reasons can be assumed to be unidirectional. Well-known examples for strongly interchanging reactions are the transaldolase and transketolase steps in the pentose phosphate pathway (Wiechert *et al.*, 1997b).

4. In a typical metabolic network consisting of glycolysis, pentose phosphate pathway, tricarboxylic acid cycle,
FIG. 3. Typical situations in which stoichiometric MFA fails: (a) parallel pathways without any related flux measurement, (b) certain metabolic cycles, (c) bidirectional reaction steps, and (d) split pathways when cofactors (gray circles) are not balanced.

Stoichiometric MFA fails if the energy metabolites ATP, NADH, NADPH, ... are balanced together with the other metabolites (Fig. 3d) (Bonarius et al., 1997). Thus, it must be assumed that all energy-producing and consuming reactions and all the conversion reactions between the energy metabolites are exactly known. On the contrary, newer results based on 13C MFA have repeatedly shown that the balances of NADPH and NADH are not closed (Marx et al., 1996), the efficiency of NADH to ATP conversion is not precisely known (Sauer and Bailey, 1999), and strong futile cycles exist in the anaplerosis (Petersen et al., 2000).

Carbon-Labeling Experiments

Recognizing these shortcomings of stoichiometric MFA it became clear that more information is required to complement the extracellular flux data. This meant the advent of 13C MFA in metabolic engineering. 13C MFA with proton NMR measurements can be seen as a direct successor of the classical 14C labeling method which was already established for quantitative flux determination in the 1980s (Blum and Stein, 1982). However, 13C MFA is nowadays far more powerful as will become clear in the following. In particular, all the shortcomings of stoichiometric MFA mentioned above have been overcome.

13C MFA is based on a carbon-labeling experiment (CLE) (Marx et al., 1996). In such an experiment a specifically 13C-labeled substrate like, e.g., [1-13C]glucose is fed to the biological system. The labeled carbon atoms are then distributed all over the metabolic network until finally the isotopic enrichment in the intracellular metabolite pools can be measured by NMR or MS instruments. The resulting data provide a large amount of additional information to quantitate the intracellular fluxes. Figure 4 summarizes the principle of 13C MFA: From measured extracellular fluxes and measured intracellular labeling information the intracellular fluxes must be computed. However, the computational procedure behind this simple “formula” is quite complicated and mathematically rather involved. Thus, it took several years until powerful computational tools for 13C MFA became available.

Isotopomers

A central concept of 13C MFA in the newer literature is that of an isotopomer of a given metabolite (Malloy et al., 1988). The term isotopomer is a combination of the terms isotope and isomer and it means one of the different labeling states in which a particular metabolite can be encountered (Figs. 5 and 6). Because a metabolite with \( n \) carbon atoms can be labeled or unlabeled at each carbon atom position, there can be \( 2^n \) different labeling states of this molecule which means that there are \( 2^n \) different isotopomers. Figure 5 shows the \( 2^3 = 8 \) different isotopomers of a metabolite with 3 carbon atoms.

The isotopomer distribution of a metabolite with \( n \) carbon atoms is characterized by the percentage of each isotopomer within the metabolite pool, i.e., the isotopomer fractions. Clearly, the isotopomer fractions for each metabolite pool must sum up to one (i.e., 100%) as illustrated in Fig. 5b. The labeling state of the cell is precisely quantitated by the isotopomer distributions of all metabolites. Observing the intracellular labeling state of a microorganism in a CLE thus requires measured quantities that are related to the isotopomer distribution.
FIG. 4. Principle of $^{13}\text{C}$ metabolic flux analysis. The intracellular forward and backward fluxes must be determined from the measured extracellular fluxes and the measured intracellular labeling information.

The fate of the isotopomers in each enzymatic reaction step is determined by the fate of the carbon atoms of each metabolite. Figure 2b shows the carbon atom transition network for a simple example. From the carbon transition network, the isotopomer network is constructed. This network describes how the various isotopomers react with each other.

Historical Remarks

Before going into the details, some milestones in the development of $^{13}\text{C}$ MFA are summarized.

1982: Already in the 1980s powerful but extremely time-consuming frameworks for MFA based on $^{14}\text{C}$ labeling were available (Blum and Stein, 1982). They have been applied for example to Tetrahymena cells.

1988: Pioneering work in isotopomer analysis was done by Malloy et al. (1988) in an investigation of the citric acid cycle activity in the heart. At this time $^{13}\text{C}$ NMR data were only obtained for glutamate and thus the analysis was rather rudimentary.

1993: The same limitations hold for the first application of MS (again with mammalian organs) (Katz et al., 1993).

1995: CLEs with $^1\text{H}$ NMR measurements are much easier to evaluate than with $^{13}\text{C}$ NMR or MS data. The first flux analysis which was based on a general mathematical modeling approach was carried out in Zupke and Stephanopoulos (1995).
FIG. 6. Typical $^{13}$C NMR spectra evolving from a metabolite with three carbon atoms. The middle carbon atom produces a singlet, two kinds of doublet, or a double doublet peak depending on the labeling state of its neighbors. The isotopomer distribution of this pool produces several superimposed spectra with different intensities from which four component spectra can be determined by deconvolution of the peak fine structures.

1996: The first extensive labeling data set with more than 25 $^1$H NMR measurements was produced and evaluated in Marx et al. (1996).

1997: General statistical evaluation procedures for $^{13}$C MFA with $^1$H NMR measurements were first elaborated in Wiechert et al. (1997b).

1997: Riboflavin production with Bacillus subtilis (Sauer et al., 1997) was the first extensive application of $^1$H/$^{13}$C 2D COSY NMR to microbial metabolism. However, powerful mathematical evaluation tools were missing at that time.

1998: The first rigorous $^1$H/$^{13}$C 2D COSY NMR data evaluation by the parameter-fitting approach was carried out for Escherichia coli in Schmidt et al. (1998).

1999: The investigation of the cellular energy metabolism is one of the most promising perspectives of $^{13}$C MFA as has been done in Sauer and Bailey (1999).

1999: The introduction of MS instruments into $^{13}$C MFA posed new mathematical problems. In Park et al. (1999), the anaplerotic fluxes of C. glutamicum are investigated and evaluated by a comprehensive yet not universal modeling approach.

1999: The comparison of different physiological states has become one of the most popular applications of $^{13}$C MFA as has been done for Zymomonas mobilis in de Graaf et al. (1999).

2000: The dynamical properties of isotopomer labeling systems have first been fully understood in Wiechert and Wurzel (2000) which led to the development of a universal and computationally efficient framework for $^{13}$C MFA.

2000: In Petersen et al. (2000), this general modeling approach was first combined with an experimental design method to resolve four forward and backward anaplerotic fluxes in C. glutamicum. A significant futile cycling was proven.

2001: Dauner et al. (2001) present the first combined evaluation of NMR and MS data for B. subtilis. The tremendous amount of 133 NMR and 468 MS signals could be consistently evaluated.

Other Literature

The major developments dating from the time before MFA entered metabolic engineering are summarized in several excellent reviews, text books, and articles (Anderson, 1983; Lambrecht and Rescigno, 1983; Blum and Stein, 1982). The principles and problems of stoichiometric MFA are described in Bonarius et al. (1997), Varma and Palsson (1994), and Vallino and Stephanopoulos (1993) and the early years of $^{13}$C MFA have already been reviewed in Stephanopoulos et al. (1998), Szyperski (1998), Wiechert and de Graaf (1996), and Christensen and Nielsen (1999b). Applications to specific microorganisms are described in de Graaf (2000a, b). An interesting comparison with tracer applications in cell physiology is presented in Kellerer (2001). This minireview concentrates on the recent methodological developments in $^{13}$C MFA. It presents the basic principles, while the details of experimental procedures,
measurement protocols, and mathematical evaluations can be found in the more advanced cited publications.

CARBON-LABELING EXPERIMENTS

Carrying out a CLE is a rather complicated procedure in which several rules must be obeyed (Marx et al., 1996).

1. It must be guaranteed that over the whole time span occupied by the experiment the system is in a stationary metabolic state with constant intracellular fluxes. In general, metabolic stationary conditions can be established in a continuous culture and can be supervised by measurement instruments like CO$_2$ gas analysis.

2. The choice of labeled substrate must be considered carefully. In the case of glucose, the predominantly used glucose isotopomers are [1-13C]glucose and fully labeled glucose which currently cost about $100 per gram. Other labeled glucose species are only available at a substantially higher cost. In principle, any mixture of [1-13C]glucose, fully labeled glucose, and unlabeled glucose can be used for a CLE. However, only recently a rational experimental design procedure became available that allows composition of an optimal mixture for a maximal information yield (see Identifiability Analysis, Optimal Experimental Design, and Model Discrimination).

3. The bioreactor should be as small as possible to keep the cost of the experiment low. On the other hand, if the reactor volume is too small, it becomes more and more difficult to maintain stable stationary conditions. Typical reactor volumes for CLEs are currently between 300 and 1000 ml.

4. The duration of the experiment is quite critical for the correct evaluation of the measured data. After switching on the labeled substrate feed, the isotopic enrichments of all the intracellular metabolite pools undergo a transient phase until finally a steady state not only of metabolic fluxes but also of isotopomer distribution is reached. Figure 5a shows a typical transient time course for the labeled compounds of a small example network. If there are extremely large pool sizes in the system like, e.g., the glutamate pool in *C. glutamicum*, the stationary time can be on the order of a bioreactor residence time.

5. In addition to the time span required for isotopic equilibrium of the intracellular intermediates, a much longer time span must be taken into account for the equilibration of labeling in the macromolecular biomass constituents (proteins, RNA, DNA, cell wall). The reason for this is that intracellular intermediates are currently not directly measured but only those biomass compounds that are synthesized from these precursors. In this retrobiosynthetic approach the labeling information is derived from macromolecular components in the cell mass like proteins, RNA and DNA, and several more compounds (Szyperksi, 1995; Eisenreich et al., 1993). To this end, the isotopic enrichment must be stationary not only in the intracellular intermediates but also in the macromolecules. Clearly, because all cells are completely unlabeled in the beginning of the CLE, it will take some time until the “early” cells are washed out of the reactor and only “late” cells with stationary labeled macromolecular compounds remain. Fortunately this wash-out effect can be compensated for (van Winden et al., 2001b). Depending on the microorganism under investigation, CLEs typically take between two and four bioreactor residence times which is the reason for a potentially high substrate cost.

6. It must be kept in mind that unlabeled and specifically labeled substrates do not really contain 100% of the nominal isotopomer. This comes from the fact that there is a natural abundance of 1.13% $^{13}$C, which means that the nominally unlabeled carbon atom positions within the particular molecule are in fact labeled with a probability of 1.13%. Consequently, any specifically labeled substrate apart from the fully labeled variants is a mixture of several different isotopomers.

7. After the experiment is completed, the cell mass is harvested by centrifugation. Afterward, the cells are disrupted, the different macromolecular components are isolated from the broth, and finally each fraction is hydrolyzed to obtain the metabolic precursors from which they are composed. With this technique, all amino acid precursors, ribose 5-phosphate, fructose 5-phosphate, and glucose 6-phosphate, can be indirectly observed. However, care must be taken that the extraction and hydrolyzation processes do not destroy the chemical compounds.

MEASUREMENT PROCEDURES

Any measurement instrument that can detect differences between isotopomers is suitable for gaining information about the intracellular labeling state of the system. Different types of NMR and MS techniques (Wittmann, 2001; Szyperksi, 1998) have been developed in order to obtain maximum information about the intracellular isotopomer distribution.

**NMR Techniques**

1. $^1$H or proton NMR was the first method which was extensively applied to $^{13}$C-labeling experiments (Marx et al., 1996). By this method, each single protonated carbon atom position inside the particular metabolite pool can be

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observed separately from the other positions. The measured information is the positional enrichment of each carbon atom position which is the percentage of isotopomers labeled at this specific position. Clearly, if there are \( n \) carbon atoms in a metabolite, this produces at most \( n \) different positional enrichment values which is only a small part of the total isotopomer information. On the other hand, pure proton NMR experiments are much easier to evaluate than experiments with more isotopomer information, which explains why \(^{13}\text{C}\) MFA was first elaborated for this type of experiment.

2. In a \(^{13}\text{C}\) NMR spectrum the isotopomer distribution is resolved in more detail because a labeled carbon atom produces different hyperfine splitting signals depending on the labeling state of its direct neighbors in the molecule (Fig. 6) (Schmidt et al., 1998). Typically, if the neighboring carbons are not labeled, a singlet peak emerges. If only one of the neighbors is labeled, a doublet peak results, where the size of the doublet splitting depends on the functional group in which the neighbor is situated. Finally, if both neighbors are labeled, a doublet of doublets results which however may degenerate into a triplet when both doublet splittings happen to be equal. Unfortunately, a completely unlabeled isotopomer produces no \(^{13}\text{C}\) NMR signal. This means that the observed NMR signals only reflect part of the isotopomer distribution and thus no percentage value can be assigned to each type of singlet, doublet, or double doublet peak. Usually, ratios of different peak areas are then taken to obtain a dimensionless quantity that does not depend on the overall signal strength.

3. In general, the relation between the molecular structure and the \(^{13}\text{C}\) NMR spectrum is more complicated for branched molecules, symmetric molecules, and molecules containing aromatic rings (Szyper, 1998). Multiply labeled aromatic compounds can give rise to second-order even higher order NMR spectra wherein disentangling of the \(^{13}\text{C}\) signals is extremely difficult. On the other hand, \(^{13}\text{C}\) NMR spectroscopy in some cases also allows the observation of long-range couplings (van Winden et al., 2001b) which increase the information content of the measurements. Thus, peak fine structures that are produced by labeled carbon atoms which are not direct neighbors in the molecular carbon backbone can be analyzed. However, such long-range couplings are observed only for a few molecules like tyrosine, histidine, and sugars.

4. A combination of \(^1\text{H}\) and \(^{13}\text{C}\) NMR is the two-dimensional NMR technique (Szyper, 1995). The advantage of this method is that, as opposed to the formerly mentioned methods, the different compounds do not have to be isolated from the hydrolysate before the measurement takes place. The reason for this is that the peaks of different metabolites are separated into a two-dimensional spectrum, and thus each spectrum can be evaluated without complex interferences from other compound spectra. On the other hand, some carbons may be unobservable in \(^1\text{H}^{13}\text{C}\) 2D COSY NMR because they have no proton attached.

Problems with NMR

NMR for MFA purposes is a rather specialized discipline which is different from other NMR applications. In particular, an NMR spectrometer is not directly available for any research team and the evaluation of NMR spectra requires some care.

1. The optimization of NMR measurement parameters and the proper interpretation of all the peak fine structures in a spectrum require a skilled NMR specialist. For certain metabolites, specialized measurement procedures may be required in order to obtain additional isotopomer information (de Graaf et al., 2000).

2. The decomposition of the entangled singlet, doublet, and triplet peaks in a spectrum and the precise quantitation of the peak areas require specially adapted software tools which are not generally available (van Winden et al., 2001b).

MS Techniques

An alternative to NMR which has been lively discussed over the past few years is mass spectrometry which tends to be much more applicable and to exhibit a much higher sensitivity than NMR. MS instruments are available in different variants. As for 2D-NMR, the MS instruments can work directly with the hydrolysate.

1. In GC–MS, the MS instrument is coupled to a gas chromatograph to separate the compounds of the hydrolysate (Christensen and Nielsen, 1999a). The compounds eluting from the GC column must be ionized which simultaneously causes a fragmentation of molecules. Thus, not only the mass isotopomers of the molecular ion are measured but also the mass isotopomer spectrum of several fragments. This significantly increases the measured information.

2. In LC–MS, the GC is replaced with a liquid chromatograph (de Graaf, 2000c). The main difference is that chemical derivatization is not necessary. An extension to this technique is an LC–MS–MS instrument with tandem mass filters. However, applications of LC–MS to MFA are still rare.

3. A promising innovation is the quantitative use of a MALDI–TOF–MS which is usually applied for protein analysis (Wittmann and Heinzle, 2001b).
In each case the MS instrument divides a particular isotopomer distribution by the molecular weights of the isotopomers (Lee et al., 1991). This means that all isotopomers of a particular metabolite containing the same number of labeled carbon atoms are summarized under one peak signal. Because every isotopomer (especially the unlabeled isotopomer) contributes to exactly one peak in the MS spectrum, a percentage value can be calculated for each peak. This yields the so-called mass isotopomer fractions. For a metabolite with $n$ carbon atoms, $n + 1$ measurements are produced. After normalization, exactly $n$ informative mass isotopomer quantities remain.

**Problems with MS**

Although the MS techniques seem to be much easier to handle than the NMR techniques, several problems with the measurement procedure occurred which may be the reason why MS is not yet as established as NMR for measurement procedure occurred which may be the reason why MS is not yet as established as NMR for $^{13}$CMFA. The following effects must be carefully taken into account and corrected. Quantitative formulas to achieve this correction are available in the literature.

1. Especially for GC-MS, the sample must be prepared by chemical derivation in order to obtain chargeable molecules. DMFDMA (Christensen and Nielsen, 2000) and MTBSTFA (Dauner and Sauer, 2001) have been used to derivatize amino acids.

2. Strong isotope effects have been observed by Dauner and Sauer (2001), which means that the retention time of differently labeled isotopomers in the GC column depends on the isotopomer. Similarly, overloading of the MS detector must be prevented according to Dauner and Sauer (2001).

3. The natural abundance of other atoms than carbon leads to a disturbance of the mass isotopomer spectrum (Fernandez and Rosiers, 1996). For example, each oxygen atom in the molecule might also be present as an $^{17}$O isotope with the probability of 0.037% and an $^{18}$O isotope with the probability of 0.204%. Much more significant is the effect of silicon having a natural abundance of 4.70% $^{29}$Si and 3.09% $^{30}$Si. Si occurs in derivatizing agents.

**EVALUATION OF CLEs**

The centerpiece of data evaluation is a simulation of the CLE (Schmidt et al., 1997). In this simulation it is assumed that the intracellular fluxes are already known. Based on guessed flux values and the known input substrate composition, the stationary distribution of isotopomers over the network can be computed. For each isotopomer in the system its isotopomer fraction relative to the corresponding metabolite pool must be determined by the simulation. Although this is only required for the stationary labeling state in which the measurements take place, the nonstationary transient can also be simulated if the metabolite pool sizes are also assumed to be known. An example of such an nonstationary transient is shown in Fig. 5a where all pool sizes are set to 1 for simplicity.

The mathematical procedure for simulating CLEs is computationally expensive (Möllney et al., 1999). This comes from the fact that the mathematical model describing the dependency between the intracellular fluxes and the stationary isotopomer distribution contains one (often nonlinear) isotopomer balance equation for each isotopomer in the system. For a realistically complex central metabolic pathway model together with the biosynthetic pathways for macromolecular precursor compounds, this gives rise to a nonlinear equation system of dimension 1,000 and greater. This is due to the fact that some large molecules like chorismate alone have 1,024 isotopomers. Several iterative algorithms for the solution of the nonlinear equation system have been suggested in the literature (Schmidt et al., 1997; Wiechert et al., 1997a) until finally an analytical solution was found based on matrix calculations (Wiechert et al., 1999). Today the computation time for one simulation run is on the order of 1 s.

**Flux Determination**

Having established a simulator for CLEs, the most widely applied evaluation algorithm proceeds as follows (Dauner et al., 2001; Schmidt et al., 1998; Wiechert et al., 1997b) (Fig. 7).

1. Guess some flux distribution over the metabolic network which fulfills the stoichiometric balance equations.

2. Simulate a CLE based on this flux distribution and the known isotopomer fractions of the input substrate.

3. From the outcomeing isotopomer distribution compute the measured values that would result if the guessed fluxes were present in the system.

4. Compute the difference between the measurements predicted by the simulation and those measurements which were actually obtained. The discrepancy is usually measured
by a sum of squares where each single residual value is weighted by the corresponding measurement standard deviation.

5. Based on the computed discrepancy, a systematic variation of the guessed fluxes is performed by applying an optimization algorithm like a simplex method, a simulated annealing algorithm, an evolutionary strategy, or a Newton-like algorithm. This implies an iteration of steps 2–4.

When using a gradient-based optimization strategy it is quite important to have the sensitivity of the isotopomer distribution with respect to the guessed fluxes. Although these sensitivities can be calculated numerically, it is much less time consuming to calculate them from an analytical formula (Wiechert et al., 1999). Today the computation time for sensitivity analysis is on the same order of magnitude as the simulation time.

Several attempts have been published in the literature to obtain a direct (i.e., noniterative) flux estimation procedure (Klapa et al., 1999; Sauer et al., 1997). In that case, derivation of simple explicit formulas from the isotopomer balance equations that relate the intracellular fluxes to the measured quantities is attempted. This makes data evaluation very simple even for the nonspecialist. However, all of those methods are limited to a certain network structure or to a certain type of labeling experiment. Moreover, they do not consider the complete measured information and thus they are suboptimal from a statistical point of view.

**Statistical Analysis**

After termination of the parameter-fitting procedure, a detailed statistical analysis of the estimated fluxes is necessary because biological systems and measurements are usually extremely noisy and thus the error propagation from the measurements to the estimated fluxes may lead to seemingly precise but statistically worthless results. The following statistical techniques have been established in recent years for isotopomer systems (Mölney et al., 1999).

1. The sensitivities of the measured values with respect to the estimated fluxes are collected in the output sensitivity matrix. This matrix shows which of the fluxes have the strongest influence on which measurements.

2. From the output sensitivity matrix the flux covariance matrix of the estimated parameters can be immediately computed. From this covariance matrix a confidence region for fluxes can be derived. In particular, a confidence interval can be assigned to each single estimated flux value.

3. Another matrix that can be directly computed from the output sensitivities is the parameter sensitivity matrix which explains how a change in a certain measurement would change the estimated fluxes. This is valuable information because it reveals the most influential measurement values.

4. As a routine procedure a \( \chi^2 \) test is applied to the obtained minimal sum of squares. By this test the goodness of fit can be judged and possible gross measurement errors can be detected and excluded from the data set.

**Identifiability Analysis, Optimal Experimental Design, and Model Discrimination**

The first ingredient of a rational design procedure for CLEs is an identifiability analysis to find out whether or not the information about the unknown fluxes is contained in the measured values. The identifiability question must be answered a priori (that is, before the CLE is actually carried out) which makes the treatment of this problem particularly difficult. Computational solutions to the identifiability problem are now available (Wiechert et al., 2001). If it turns out that the fluxes are not identifiable, the experiment is
badly designed and thus more measurements are required, or otherwise the network needs to be simplified.

The optimal design of a CLE is the next step which has been addressed only recently (Möllnay et al., 1999). Having resolved the identifiability problem, the question now is how to compose the input substrate mixture for the CLE in order to obtain maximal flux information. Clearly this question depends on the aim of the CLE and the magnitude of the measurement noise. If only a certain part of the metabolic network is in the focus of interest, the other fluxes need not be precisely estimated. Thus, the design of a CLE requires a proper weighting of the different fluxes in the system to specify its aims. If this has been done, different input mixtures can be quantitatively compared for some guessed flux distribution and an optimal mixture with respect to chosen criteria can be computed.

Although the central metabolic pathways are well known, the network models used in the literature differ. The reason is that simplifications must be made in the case of nonidentifiable fluxes. For example, pools are lumped or unidirectionality assumptions must be made (Wiechert et al., 2001). In some cases, a pathway or reaction may assumed to be inactive or not expressed (Klapa et al., 1999). Moreover, unknown pathways or metabolic channels may be present (van Winden et al., 2001a). This leads to the problem of model discrimination which can be solved based on the \( \chi^2 \) criterion to judge how well a model fits (Dauner et al., 2001; Klapa et al., 1999).

### Software Frameworks

The evaluation of a CLE cannot be managed without a software system that takes care of all the measured data, automatically generates the model equations, enables simulation and sensitivity analysis, and computes the flux estimates and statistically evaluates them. Several systems have been developed in the literature which are distinguished by their degree of specialization, the modeling approach, ease of use, requirement of programming work from the user, performance of the algorithms, and extent to which all mentioned problems can be solved. Only some typical systems shall be mentioned.

1. In Schmidt et al. (1998), the simulation of isotope-pomer distributions and parameter fitting was achieved for the first time. The system is based on MATLAB and thus can be used and extended by an experienced MATLAB programmer.

2. The approaches of Klapa et al. (1999) and Sauer et al. (1997) are based on explicit formulas for flux determination. Thus, they are limited to a certain network topology and do not make use of the full measurement information.

On the other hand, they are simple and can be applied very quickly.

3. A completely general software which solves all the mentioned problems above is described in Wiechert et al. (2001). However, a rather large software system came out and the price for generality is that the proper specification of an experiment requires considerable configuration data.

In any case, the programs cannot be used without a rough understanding of the underlying mathematical concepts. Due to the significant biological and instrumental problems in the process of MFA, a completely automated flux analysis strategy is still an illusion.

### Future Developments

During recent years \(^{13}\)C MFA has reached a state of relative maturity. The experiments themselves have become a routine procedure, the measurement techniques are well established, and sophisticated mathematical evaluation algorithms are available. However, there are still some open problems and several perspectives for future development. The following aspects are currently under discussion.

### Applications

1. The application of \(^{13}\)C MFA to eukaryotes (Christensen et al., 2000; Maheimo et al., 2001) or even plant and animal cells (Roscher et al., 2000) is still extremely difficult. This comes from the intracellular compartmentation that must be properly modeled in the network model. For example, oxalo acetate is a metabolite that can be found in the cell cytosol and in the mitochondria as well. The current experience is that MFA models for eukaryotes do not fit the measured data as well as for prokaryotes.

2. In a cell culture the growth rate can be neglected which means that labeling information cannot be obtained from cell components. In this situation labeled products and by-products must be measured which is a significantly reduced amount of information and requires properly simplified network models in order obtain identifiable fluxes.

3. It is still an open question to what extent the metabolism of single cells within a continuously growing population is different. This is definitely true for hyphenating fungi and some animal cell cultures. While the basic assumption of MFA is that this population variety is not the case, this effect may lead to a misinterpretation of data even though the model possibly fits well. However, the development of single-cell measurement techniques is only at its beginning (Durack and Robinson, 2000).
Experimental Procedures

1. One of the major challenges for $^{13}$C MFA is the application to nonstationary processes. In particular, the analysis of batch-fed processes is of great importance for practical industrial applications (Wittmann and Heinzle, 2001).

2. The direct measurement of intracellular intermediates without taking the retrobiosynthetic approach would tremendously accelerate the procedures.

3. Another relevant application is the screening of large numbers of microbial strains. In this case, a rudimentary flux analysis might already yield enough information to distinguish between different strains. On the other hand, the CLE must be miniaturized, the measurement procedure must be accelerated, and the experiment evaluation must be automated (Wittmann et al., 2001).

Spectral Deconvolution

1. The quantitative evaluation of entangled NMR spectra is very important for MFA. However, this is still rather an art than a rigorous method. In particular, the assignment of measurement errors to peak areas must be based on a strictly rational procedure (van Winden et al., 2001b; Wittig et al., 1995).

2. Likewise for the evaluation of biological MS spectra it is an open question of how precise an MS instrument really is. Due to the disturbances mentioned above, development of a rigorous procedure for spectral evaluation and error estimation is only at its beginning (Dauner and Sauer, 2001).

Data Evaluation

1. The development of a user interface for MFA that facilitates the specification of an experiment certainly is an important task for the routine industrial application of $^{13}$C MFA (Wiechert et al., 2001).

2. CLEs and their evaluation produce large amounts of data that are difficult to interpret from the computed matrices and tables. Extensive visualization of networks, data, and statistical quantities is an important tool to obtain a quick understanding of the results for a mathematically unskilled person (Buschmann et al., 2000).

3. The evaluation of CLEs is one of the most complicated mathematical methods ever applied to biological systems. The general acceptance of this method and the understanding of a rigorous mathematical approach can be promoted by interactive teaching environments (Wiechert, 2001).


