Research Report

Ion-Trap Mass Spectrometry Used in Combination with Gas Chromatography for High-Resolution Metabolic Flux Determination

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ABSTRACT

Metabolic fluxes provide a detailed metric of the cellular metabolic state. Fluxes are estimated indirectly from available measurements, and various methods have been developed for this purpose. Of particular interest are methods that make use of stable isotopic tracers because they enable flux estimation at a fine resolution. In this report, we present a protocol for the use of ion-trap mass spectrometry (MS) in combination with gas chromatography to measure the mass isotopomer distribution of biomass hydrolysates. At physiological steady-state, these measurements directly reflect the isotopic tracer distribution in the amino acid central carbon metabolism precursors. Because the extent to which a metabolic flux network can be accurately resolved strongly depends on the reliability and precision of the MS measurements and, in light of the current need for quantitative high-throughput biological analysis at the microscale, we discuss every step of the measurement process, indicate possible sources of error, and suggest solutions to avoid them. Potential advantages to using ion-trap versus quadrupole MS are also addressed. The final protocol requires

0.5 mg of dry biomass to detect the mass isotopomer distribution of 2–4 fragments of 13 amino acids, with a relative variance less than 1% for the most abundant peaks.

INTRODUCTION

The post-genomic era has placed increasing demands for accurate, reproducible, and noninvasive measurement methods to elucidate cellular physiology in a quantitative manner. Additionally, it should be possible to extend such methods to high-throughput applications so that sufficient data can be collected under a variety of genetic and environmental conditions. This necessitates the decrease of the initial sample size to microscale or nanoscale levels, which raises the question of whether existing techniques are adequate for this purpose. Thus, it is essential to study available measurement methods to assess their reliability, accuracy, sensitivity, scalability, and measurement redundancy because these attributes directly affect the quality of the observability of a particular cellular system.

In this paper, we study the use of ion-trap mass spectrometry (MS) in combination with gas chromatography (GC) for the measurement of the ¹³C mass isotopomer distribution of biomass hydrolysates (1) in the context of metabolic flux analysis (see References 2–4 for definition of “flux” and details about the importance of flux quantification). The use of mass isotopomer distribution measurements of

biomass hydrolysates for flux determination of entire metabolic networks was illustrated for the first time by Christensen and Nielsen (5,6). Subsequent studies (7–10) used the method for the analysis of a variety of metabolic flux networks. Yet there are still issues concerning the experimental setup and protocol that need to be clearly resolved because they might strongly affect flux quantification (6,11). These issues concern the selection of the derivatization agent, the sources of error in the various stages of the measurement process and how they might affect the final outcome, the size of the initial sample, the peak resolution in the mass spectrum, the precision, accuracy, and reproducibility of MS measurements, and the validation of metabolic and isotopic steady-states. Here we present in detail the development of an experimental protocol for the measurement of the ¹³C mass isotopomer distribution of biomass hydrolysates of *Corynebacterium glutamicum* while addressing sequentially all these issues and provide adequate information for the methodology to be adapted to any other cellular system. In addition, since we are the first to report the use of ion-trap MS for the measurement of the mass isotopomer distribution of biomass hydrolysates, we also discuss observed problems, which originate primarily from saturation phenomena in the ion trap and are thereby unique to the ion-trap MS users, and suggest solutions to avoid them. Finally, we address the issue of

sensitivity, specifically how the higher sensitivity of the ion-trap compared to the quadrupole mass spectrometer enables the accurate quantification of mass isotopomer distributions with fewer runs and from smaller initial sample sizes, providing a potential advantage for the use of ion-trap MS for measurements at the microscale.

MATERIALS AND METHODS

The gas chromatograph- (ion-trap) mass spectrometer used in this study was a Varian Saturn 2000 (Varian, Palo Alto, CA, USA). The experiments were carried out at electron ionization (EI) mode at 70 eV. The samples were injected automatically at 1 μ L sample per injection. The injection mode was splitless unless otherwise specified.

The biomass samples used in the development of the experimental protocol were obtained from shake flask and chemostat experiments of *C. glutamicum* ATCC 21799 under glucose limitation (11,12). The protocol presented is based on the biomass composition of *C. glutamicum*. However, the details provided within the text about the methodology are adequate to allow for the adaptation of the protocol to any other metabolic system.

EXPERIMENTAL PROTOCOL OPTIMIZATION

When measuring the mass isotopomer distribution of biomass hydrolysates for flux quantification, the structure and, consequently, the frag-

mentation pattern, and the concentration of the compounds in the analyzed mixture (i.e., amino acids in biomass) are known. In this case, it is essential to appropriately adjust the experimental protocol so that all amino acids elute separately from the gas chromatograph; the mass spectrometer accurately resolves masses differing by one mass unit (Da) in the entire range of amino acid molecular weights, multiple fragments of all, if possible, amino acids are measurable, and reproducible measurements of their mass isotopomer fractions are obtained. Calibrating just the GC-MS equipment is insufficient for meeting all these requirements. Measuring the mass isotopomer distribution of biomass hydrolysates is a multistage procedure (Figure 1), and the optimization of each individual step is required.

Metabolic and Isotopic Steady-State

To directly use the mass isotopomer measurements of biomass hydrolysates in metabolic flux quantification, metabolic and isotopic steady-state must be guaranteed by all macroscopic data. Only at these physiological steady-state conditions, the isotopic tracer distribution of the amino acids in the biomass directly reflects this of their central carbon metabolism precursors according to the stoichiometry of the amino acid biosynthetic pathways. While the isotopic measurements of biomass hydrolysates can also be used in the analysis of metabolic and/or isotopic transient systems, at these physiological conditions, however, the mapping of the isotopic tracer distribution

Figure 1. The measurement of the mass isotopomer distribution of biomass hydrolysates is a multistage procedure.

of central carbon metabolism precursors into this of the amino acids in biomass is not obvious and has to be accurately resolved before the biomass isotopic measurements can be used to derive conclusions about the cellular flux distribution. Specifically, at metabolic steady-state but isotopic transient systems, the direct mapping of the isotopic distribution of central carbon metabolism metabolites into that of amino acids in the biomass is “skewed” by the sizes of the intermediate metabolite pools, while at metabolic transient conditions, it also depends on the rate of change of the intermediate metabolite pool sizes.

Determination of Biases in the Measurements

For this application there is no suitable internal standard, because it has to be one amino acid but still reflect the systematic biases affecting the mass isotopomer fraction measurements of each amino acid, independently of its size and complexity. Any systematic detection error in the measurements was then determined by the comparison of the measured mass isotopomer fractions from a large set of single amino acid and biomass hydrolysate samples at natural abundance with their theoretically expected values. Labeled glutamate sam-

ples [(1-¹³C) and (U-¹³C)] of known label enrichment were also measured. It is then assumed that if the exact same protocol is followed (including the fine tuning of the GC-MS equipment), the mass isotopomer measurements will always be as precise. It was observed that the difference between the theoretical and the measured values was larger than the variation between various injections and duplicate samples for mass isotopomer fractions corresponding to peak areas smaller than 200 counts (see Figure 3 for such an example) and for most of the mass isotopomer fractions of heavier and more complex than glutamate amino acids. We believe that, in the first

Figure 2. Plots of the measured peak areas of (M+0), (M+1), (M+2), and (M+3) mass isotopomers of fragment 316 (= M) of TBDMS-aspartate (ASP316) versus the total measured peak area of ASP316 from five injections of a 4-mg/mL aspartate sample at natural abundance. Linear and second order polynomial fits of the measured values are shown by the dotted lines. The slope of the solid line is equal to the theoretically expected value for the depicted mass isotopomer fraction. The observed deviation from linearity indicates the occurrence of saturation phenomena in the ion trap.

case, the strong biases are due to the fact that the measurements are at the detection limits of the technique, while we speculate that, in the second case, they are primarily due to the tuning of the GC-MS equipment, which was focused on the lighter and less complex amino acids. In any case, when such detection errors, which cannot be accounted for by the variance between multiple injections and duplicate samples, are identified at the calibration stage, and it is not possible to eliminate them by modifying the experimental protocol, they always have to be taken into consideration in the error assigned to the measurements of the particular isotopomer fractions. More-

over, the high degree of redundancy in the MS measurements proves beneficial for validating the obtained measurement values and the standard deviations assigned to them (11).

Hydrolysis

Biomass hydrolysis involves the treatment of a biomass pellet with HCl at high temperatures ($>90^{\circ}\text{C}$) for a certain amount of time (1). The selected combination of HCl concentration, temperature, and hydrolysis time should allow for the completion of the amino acid extraction but prevent the thermal decomposition of unstable

amino acids (see References 1, 5, 7–10, 13 for various hydrolysis protocols). After deciding to use 6 M HCl to hydrolyze biomass at 120°C , we selected a hydrolysis time of 20 h after studying the mass spectra obtained at 16, 20, and 24 h. At this hydrolysis time, it was occasionally possible to detect cysteine and histidine, when single amino acids were used, while these amino acids were not observable in the spectra of the other hydrolysis times.

Selection of Derivatization Agent

GC analysis is limited to compounds that are volatile or can be

Figure 3. Plots of the measured peak areas of (M+0), (M+1), (M+2), and (M+3) mass isotopomers of fragment 418 (= M) of TBDMS-aspartate (ASP418) versus the total measured peak area of ASP418 from five injections of a 4-mg/mL aspartate sample at natural abundance. There is no indication of saturation phenomena influence on these measurements because they can be best fitted by a straight line (dotted line). The slope of the solid line is equal to the theoretically expected value for the depicted mass isotopomer fraction, while the difference between the two slopes is equal to the detection bias for this mass isotopomer fraction (detection biases are pronounced for peak count numbers smaller than 200).

made volatile and are sufficiently stable to be run through the (heated) GC column (14). Derivatization can be used to increase the volatility and stability of some compounds, amino acids included. There is a variety of derivatization agents for amino acids reported in the literature (5,10,14–17). In the context of metabolic flux analysis, the key criterion for selecting a particular derivatization agent should be the number and kind of fragments that are measurable for each detected compound because it is fragmentation that enhances the value of MS as a flux analysis tool (5,6,11,12). In this context, the derivatization methods used by Christensen and Nielsen (5,10), for example, and the one leading to tert-butyl-dimethyl silyl (TBDMS) derivatives used in the present work are equivalent (compare Table 1 with the corresponding tables of References 5 and 10). A slight advantage of the latter over the former is that the fragmentation pattern of the TBDMS amino acid derivatives is available in the literature (see, for example, Reference 14) and incorporated in the main databases used by the analysis software of all commercially available mass spectrometers.

Avoiding Saturation Phenomena—
Linear Range of Ion-Trap Function

The main structural difference between the ion-trap mass spectrometer and the quadrupole analyzer is that the ion trap is simultaneously the ion source and the mass analyzer. This configuration is beneficial because it increases the sensitivity of the ion trap over that of the quadrupole because all generated ions “reach” the mass analyzer, which allows for high resolution spectra to be obtained with lower sample concentrations. It is, however, this same nature of the ion-trap MS that generates problems that are not encountered with quadrupole analyzers. In the ion trap, saturation phenomena identified as ion/neutral molecule reactions (18,19) or space-charge effects (19–21) can occur, causing a loss of quantitative response. These phenomena have been the main reason why the ion trap is not (yet) popular among the mass spectrometer users for quantita-

Table 1. The Measured Mass Isotopomer Fractions of Eight TBDMS-Amino Acid Derivatives from the Biomass Hydrolysates of a Chemostat *C. glutamicum* Culture Operating at 0.1 h⁻¹ at Glucose Limitation after the Introduction of 50% (1-¹³C) Glucose

Fragment	Mass Isotopomer	Measured Fraction	sd
Alanine 260 [1-2-3]	M+0	60.43%	± 0.20% (0.33%)
	M+1	26.81%	± 0.54% (2.01%)
	M+2	9.73%	± 0.26% (2.67%)
	M+3	2.55%	± 0.25% (9.80%)
	M+4	0.47%	± 0.27% (57.45%)
Alanine 232 [2-3]	M+0	63.00%	± 0.21% (0.33%)
	M+1	25.93%	± 0.12% (0.46%)
	M+2	8.688%	± 0.18% (2.07%)
	M+3	2.06%	± 0.12% (5.83%)
	M+4	0.32%	± 0.01% (3.13%)
Glycine 246 [1-2]	M+0	74.99%	± 0.83% (1.11%)
	M+1	16.57%	± 0.81% (4.89%)
	M+2	7.09%	± 0.39% (5.50%)
	M+3	1.32%	± 0.59% (44.7%)
	M+4	0.02%	± 0.04% (200.0%)
Glycine 218 [2]	M+0	74.97%	± 1.63% (2.17%)
	M+1	16.18%	± 0.75% (4.64%)
	M+2	6.94%	± 0.51% (7.35%)
	M+3	1.44%	± 0.26% (18.06%)
	M+4	0.47%	± 0.31% (65.96%)
Valine 260 [2-3-4-5]	M+0	50.70%	± 0.7% (1.38%)
	M+1	32.72%	± 0.55% (1.68%)
	M+2	12.24%	± 0.22% (1.80%)
	M+3	3.50%	± 0.25% (7.14%)
	M+4	0.73%	± 0.13% (17.81%)
	M+5	0.11%	± 0.07% (63.64%)
Valine 288 [1-2-3-4-5]	M+0	51.39%	± 0.65% (1.26%)
	M+1	32.68%	± 1.19% (3.64%)
	M+2	12.16%	± 0.87% (7.15%)
	M+3	3.31%	± 0.50% (15.11%)
	M+4	0.46%	± 0.29% (63.04%)
Valine 186 [2-3-4-5]	M+0	55.96%	± 0.53% (0.94%)
	M+1	30.69%	± 0.64% (2.09%)
	M+2	9.66%	± 0.49% (5.07%)
	M+3	2.90%	± 0.39% (13.45%)
	M+4	0.59%	± 0.265% (44.92%)
	M+5	0.20%	± 0.17% (85.00%)
Valine 302 [1-2]	M+0	64.04%	± 0.22% (0.34%)
	M+1	24.71%	± 0.20% (0.81%)
	M+2	9.00%	± 0.65% (7.22%)
	M+3	1.99%	± 0.38% (19.10%)
	M+4	0.14%	± 0.25% (178.57%)
Glutamate 432 [1-2-3-4-5]	M+0	40.83%	± 0.30% (0.73%)
	M+1	36.99%	± 4.29% (11.6%)
	M+2	16.77%	± 0.14% (0.83%)
	M+3	4.22%	± 2.65% (62.8%)
	M+4	0.945%	± 1.34% (141.8%)
Glutamate 272 [2-3-4-5]	M+0	51.24%	± 1.21% (2.36%)
	M+1	31.71%	± 1.41% (4.45%)
	M+2	12.69%	± 0.41% (3.23%)
	M+3	3.58%	± 0.40% (11.17%)

Table 1. Continued.

Fragment	Mass Isotopomer	Measured Fraction	SD
Aspartate 418 [1-2-3-4]	M+0	47.18%	± 0.78% (1.65%)
	M+1	32.60%	± 0.68% (2.09%)
	M+2	14.44%	± 0.995% (6.89%)
	M+3	4.69%	± 0.31% (6.61%)
	M+4	0.94%	± 0.38% (40.43%)
	M+5	0.15%	± 0.12% (80%)
Aspartate 390 [2-3-4]	M+0	50.35%	± 1.65% (3.28%)
	M+1	32.55%	± 1.41% (4.33%)
	M+2	14.52%	± 1.14% (7.85%)
	M+3	2.46%	± 1.88% (76.42%)
	M+4	0.12%	± 0.23% (191.67%)
Aspartate 316 [2-3-4]	M+0	56.65%	± 2.02% (3.57%)
	M+1	32.24%	± 1.20% (3.72%)
	M+2	8.72%	± 1.765% (20.24%)
	M+3	2.38%	± 0.84% (35.29%)
Lysine 431 [1-2-3-4-5-6]	M+0	40.44%	± 3.025% (7.48%)
	M+1	34.83%	± 0.95% (2.73%)
	M+2	18.29%	± 2.88% (15.75%)
	M+3	5.43%	± 1.22% (22.47%)
	M+4	0.97%	± 0.95% (97.94%)
Lysine 272 [2-3-4-5-6]	M+0	46.83%	± 1.98% (4.23%)
	M+1	32.97%	± 1.53% (4.64%)
	M+2	14.53%	± 0.85% (5.85%)
	M+3	4.80%	± 0.53% (11.04%)
	M+4	0.84%	± 0.49% (58.33%)
Phenylalanine 336 [1-2-3-4-5-6-7-8-9]	M+0	44.96%	± 2.92% (6.49%)
	M+1	36.02%	± 2.50% (6.94%)
	M+2	14.745%	± 2.78% (18.85%)
	M+3	3.58%	± 1.09% (30.45%)
	M+4	0.06%	± 0.13% (216.67%)
Phenylalanine 308 [2-3-4-5-6-7-8-9]	M+0	44.105%	± 1.26% (2.86%)
	M+1	34.795%	± 1.50% (4.31%)
	M+2	15.56%	± 1.00% (6.43%)
	M+3	4.59%	± 0.19% (4.14%)
	M+4	0.94%	± 0.56% (59.57%)
Phenylalanine 234 [2-3-4-5-6-7-8-9]	M+0	50.34%	± 2.17% (4.31%)
	M+1	35.56%	± 1.83% (5.15%)
	M+2	11.96%	± 0.50% (4.18%)
	M+3	2.065%	± 1.74% (84.26%)
	M+4	0.09%	± 0.17% (188.89%)
Phenylalanine 302 [1-2]	M+0	71.93%	± 1.28% (1.78%)
	M+1	19.89%	± 1.20% (6.03%)
	M+2	7.09%	± 0.74% (10.44%)
	M+3	0.78%	± 0.49% (62.82%)
	M+4	0.04%	± 0.08% (200%)
The depicted values characterize the metabolic and isotopic steady-state of the system. The part of the amino acid carbon skeleton included in each fragment is depicted in the first column of the table under the molecular weight of the fragment. The absolute standard deviation associated with each measurement is shown in the last column of the table, while the number in parenthesis depicts the relative standard deviation.			

tive isotopic tracer analysis. However, considerable recent improvements in the technology and careful tuning, as will be discussed in the next paragraph, enhance the importance of ion-trap MS in the quantitative analysis of biological systems, in light of all its significant advantages (14).

Based on observations from varying concentration single amino acids at natural abundance (i.e., 2, 4, 6, and 8 mg/mL), saturation phenomena, in the form of excessive $[M+1]^+/M^+$ ratio, were encountered in the spectrum of at least one fragment of most of the amino acids after a certain threshold of total counts. The number of total counts is a measure of the density of the particular fragment ions in the ion trap. Excessive $[M+1]^+/M^+$ ratios have also been observed in mass spectra from quadrupoles (22) but at higher concentrations. M^+ depicts the fraction of a fragment ion pool with all its atoms in their naturally most abundant isotopic form. We also observed that the saturation threshold depended on the structure of the amino acid. Aspartate and glutamate were observed to be more prone to these phenomena than, for example, the lighter and less structurally complex alanine, glycine, and valine (data not shown). This is consistent with the fact that the probability of occurrence of ion-molecule reactions, which are among the causes of saturation phenomena in the ion trap, depends on the ion and neutral molecule structures (19). While the number of total ion counts is not directly proportional to the concentration of amino acid in the initial sample, a higher number of total counts is clearly expected at higher concentrations. Therefore, information about the saturation threshold can be used to appropriately select the amino acid derivative concentration in the original sample or the injection split ratio so that saturation phenomena are avoided. According to our observations, a concentration of 1–2 mg/mL was considered optimal; the lower bound valid for aspartate and glutamate.

It is important to recognize the presence of saturation artifacts in mass spectra, because they can significantly distort the quantification and reproducibility of the mass isotopomer mea-

surements. We present a method for the detection of these artifacts, which is independent of the mass isotopomer distribution of the measured fragments, using as examples the measured mass spectra of fragments 316 and 418 of the TBDMS-aspartate derivative (Figures 2 and 3) at natural abundance. Specifically, the fraction of the k -th mass isotopomer of an amino acid fragment is measured as the ratio of its peak area (in counts) to the sum of the peak areas of all the mass isotopomers of this fragment (referred as total counts). If no saturation phenomena occur in the ion trap, then the measured fraction of the k -th mass isotopomer should always be the same, independent of the amino acid concentration in the original sample and the ion density in the ion trap. This implies that, when plotting the peak area (in counts) of the k -th mass isotopomer against the total counts for this fragment for different sample concentrations and injections, the result should be a line with a slope equal to the fraction of the k -th mass isotopomer of the fragment. From the plots of the most abundant mass isotopomer fractions of TBDMS-aspartate fragments 316 and 418 (Figures 2 and 3, respectively) fitting five injections of a 4-mg/mL aspartate sample at natural abundance, the following can be observed. (i) Saturation phenomena are detectable in the mass spectrum of fragment 316 of aspartate. The equipment was clearly operating out of its linear range of detection for at least two of five injections. The measurements from these injections should be disregarded. If the phenomena are detected in all injections, then apparently the concentration of the amino acid in the sample should be decreased or the injection split ratio be increased. (ii) No saturation phenomena are detectable in the spectrum of fragment 418, which illustrates that such phenomena may only occur for some fragments of an amino acid derivative. This can be partly explained from the different structure of the various fragments, which might favor ion/molecule reactions. Data from all the measurable fragments of all examined single amino acid samples at natural abundance (data not shown) indicated that it is the low molecular weight fragments of an amino

acid derivative that were more prone to saturation phenomena, and aspartate fragments 316 and 418 provide such an example. This observation is consistent with the fact that the low molecular weight fragments are also subject to space-charge phenomena in the ion trap (19). (iii) Large differences in the number of total counts that correspond to a particular fragment might be observed between the various injections of the same sample (i.e., same amino acid concentration in the initial sample). Such differences imply the saturation of the ion trap from ions and neutral molecules remaining from previous injections and support the statement that the number of total counts is not directly proportional to the concentration of amino acids in the initial sample. Regular cleaning of the ion trap after a small number of runs is therefore required. In our experiments, we were running the automatic cleaning protocol of the ion trap in the beginning of each day of runs. Observations from a large number of measured samples indicated that, in the case of the GC-MS used in the present work, it was usually the even-numbered injections (e.g., the

second, the fourth, etc.) that corresponded to a high number of total counts, and thus saturation phenomena were more often detected in their spectra. Based on this (entirely empirical) observation, 3–5 injections per sample were considered optimal, expecting that at least two will be in the linear range of detection of the equipment.

Correcting for $(M-n)^+$ Peaks

Another interesting observation in the ion-trap mass spectra of the TBDMS-amino acid derivatives at natural abundance was the presence of peaks corresponding to mass isotopomers by one, two, and least often three atomic units lighter than the molecular weight of the measured fragment (in the rest of the text, they will be referred as $(M-n)^+$ peaks). Based on quadrupole MS literature of TBDMS-amino acid derivatives (23), while $(M-n)^+$ peaks might be present in the quadrupole mass spectra, they are generally negligible compared to the most abundant peaks and thereby correction of the mass isotopomer measurements to account for $(M-n)^+$ peaks is not generally

Figure 4. Schematic of the correction method for the $(M-n)^+$ peaks. Usually, the observed mass spectrum of a fragment in ion-trap MS resembles the one shown in panel B, in which molecules lighter than the molecular weight of the fragment are detected (to simplify, the lightest and heaviest mass isotopomer depicted are $M-2$ and $M+3$, respectively). Assuming that the molecules might lose hydrogen atoms during electron bombardment and defining α and β the probability for each mass isotopomer fraction to lose one or two protons, respectively, then the theoretically expected mass spectrum (corrected) shown in panel A can be estimated from the experimentally observed one.

required. However, in the ion mass spectra of the TBDMS-amino acid derivatives at natural abundance, the size of the observed (M-n)⁺ peaks is usually comparable to that of the M+3 and M+4 peaks. Therefore, there is a need to investigate to what extent ignoring them might affect the quantification of the mass isotopomer fractions. The most plausible explanation for the presence of (M-n)⁺ peaks is that ions lose one or more hydrogen atoms during electron bombardment. Chapman (18) reports this phenomenon for the reagent gas ions in chemical ionization mode. This hypothesis can also explain why the phenomenon is more pronounced in the ion trap, where the confinement conditions favor such fragmentation. Based on this assumption, we propose the following method to correct the mass spectra for the presence of (M-n)⁺ peaks.

Let us consider fragment K of a par-

ticular amino acid derivative. Figure 4B shows the measured mass spectrum [to simplify, the lightest and the heaviest measured mass isotopomers are the (M-2) and (M+3), respectively], while Figure 4A illustrates the mass spectrum if no hydrogen fragmentation had occurred ("corrected"). If α or β is the probability that an ion of fragment K, independent of labeling, might lose one or two hydrogen atoms, respectively, during electron bombardment, then, the relationships between the mass isotopomers peak area of the corrected (see asterisk below) and measured (see subscript m) mass spectra can be written as follows:

$$\begin{aligned} (M-2)_m &= \beta (M+0)^* & [\text{Eq. 1}] \\ (M-1)_m &= \alpha (M+0)^* + \beta (M+1)^* \\ (M+0)_m &= (1-\alpha-\beta) (M+0)^* + \\ &\quad \alpha (M+1)^* + \beta (M+2)^* \\ (M+1)_m &= (1-\alpha-\beta) (M+1)^* + \\ &\quad \alpha (M+2)^* + \beta (M+3)^* \end{aligned}$$

$$(M+2)_m = (1-\alpha-\beta) (M+2)^* + \alpha (M+3)^*$$

$$(M+3)_m = (1-\alpha-\beta) (M+3)^*$$

Because no mass isotopomers heavier than (M+3) were measured, any corrected peaks corresponding to mass isotopomers heavier than (M+3) are neglected.

Figure 5B shows the values of α , β , and γ (γ is the probability for an ion to lose three hydrogen atoms) estimated from spectra of the most abundant fragments of selected amino acids lighter than glutamate, based on Equation 1. They correspond to the average value of five injections of a 4-mg/mL single amino acid sample at natural abundance. Figure 5A shows the total counts measured for each fragment (the counts for the "M-n" peaks included) averaged over the five injections. According to the graphs in Figure 5, "hydrogen fragmentation" appears to be primarily a function of the structure of

a fragment ion and not of the total counts. For example, the values of α and β for fragment glycine 246 were among the largest, even though the total peak area corresponding to glycine 246 was among the smallest. Additionally, the values of α , β , and γ of glycine 246, leucine 302, methionine 234, phenylalanine 308, aspartate 302, cysteine 406, and glutamate 330 were different, even though the number of total counts was of similar size. The values of α , β , and γ for all fragments were smaller than 4%, except for glycine 246, whose α value was approximately

5.5%. These values were in agreement with the results from three other concentrations of single amino acid samples and from biomass samples at natural abundance (data not shown), indicating that the areas of the (M-n)⁺ peaks are expected to be in the order of 3% to 5% of the most abundant peak. When α and β were in this value range, the corrected mass isotopomer fractions of an amino acid fragment, estimated as the ratio of the corrected peak areas (Equation 1) to the total counts for this fragment [(M-n)⁺ peak areas included], were statistically identical to

the non-corrected mass isotopomer fractions, estimated as the ratio of the measured peak areas (Equation 1) to the total counts for the fragment after ignoring the (M-n)⁺ peak areas. The error associated with ignoring the (M-n)⁺ peaks is indeed smaller than the variance of the measurements among various injections and duplicate samples.

Biomass Composition

As previously explained, the concentration of an amino acid in the sample to be derivatized has to be appropriately selected to avoid saturation phenomena from occurring in the ion trap without affecting the accuracy of the MS measurements. Therefore, the composition of biomass has to be taken into account when optimizing a GC-(ion-trap) MS protocol for the measurement of the mass isotopomer distribution of biomass hydrolysates. Specifically, in the case of *C. glutamicum*, which is used as the model system in this study, the amino acids can be divided into three sets with respect to their concentration in the biomass as previously reported (24). The first comprises the most abundant glutamate and aspartate (glutamate accounts for approximately 50% of the *C. glutamicum* biomass), the least abundant amino acids, tyrosine, tryptophan, histidine, and cysteine, constitute the second, while the third consists of the rest. Note that the concentration of the least abundant amino acid in the second set is 50 times smaller than that of glutamate, while the average concentration of the third set is about 10 times smaller than that of the first. Due to these large concentration differ-

Figure 5. The extent of “hydrogen fragmentation” leading to (M-n)⁺ peaks differs between the amino acids and the various fragments of each amino acid. (A) Total counts measured for each of the depicted TBDMS-amino acid fragments averaged over five injections of a 4-mg/mL amino acid sample at natural abundance (each amino acid was measured separately). (B) The values of α , β , and γ (see definitions in Figure 4 legend and text) for each of the depicted fragments.

ences among the three sets, one expects that more than one split ratio is needed for the measurement of all amino acids in a *C. glutamicum* biomass sample. *C. glutamicum* (2 mg/mL) biomass samples at natural abundance were analyzed in splitless mode and split ratios of 1:10, 1:50, and 1:100. It was observed that: (i) along with arginine, asparagine, glutamine, tryptophan, and cystine, which were not detectable even when the respective single amino acid sample was measured, the peaks of cysteine and tyrosine could not be uniquely identified in the chromatogram even in splitless mode, most probably (at least for tyrosine) due to coelution. Very small peaks of cysteine (compared to the initial concentration of the sample) and only the unlabeled mass isotopomer ion of at a maximum of two fragments had been occasionally observed only in 8 mg/mL cysteine samples at natural abundance. However, the mass isotopomer distribution of 2–3 tyrosine fragments was measurable in all pure tyrosine samples examined (2–8 mg/mL). As previously reported (1), during hydrolysis, asparagine and glutamine get lost due to deamidation, while cysteine and tryptophan get lost due to oxidation. (ii) Very small peaks of histidine and only the unlabeled mass isotopomer ion of some fragments were occasionally observed in biomass samples run in splitless mode. (iii) Aspartate and glutamate measurements were subject to saturation phenomena if a split ratio higher than 1:100 was used, while the spectra of few amino acids in the third set occasionally indicated the occurrence of such phenomena when the split ratio was equal to or higher than 1:10.

Based on these observations, two runs per each biomass sample, at split ratios of 1:50 and 1:100, are considered necessary for the accurate measurement of amino acid sets 3 and 1, respectively. Because in the ion-trap mass spectrometer, the mass spectrum of all amino acids is obtained at each run, the mass isotopomer fractions of the most abundant amino acids of group 3 can also be measured from the mass spectrum at split ratio 1:100, increasing the confidence in the validity of these data. The amino acids of

set 2 are all unobservable. Since tryptophan and cystine, whose GC elution time is longer than 40 min, are unobservable, each GC-MS run is set to last for 40 min.

GC-(ION-TRAP) MS PROTOCOL

The described protocol has been tailored to measure the mass isotopomer distribution of *C. glutamicum* biomass hydrolysates. The same protocol might be applied in another metabolic system, after sample concentrations and split ratios are modified accordingly to account for differences in biomass composition among the various biological systems.

(i) Biomass samples are extracted from the reactor. (ii) The samples are centrifuged at 7000 rpm (5040× *g*) for 10 min, the supernatant is removed, and the pellets are kept at -20°C. (iii)

The pellet is then hydrolyzed with 700 µL 6 M HCl at 120°C for 20 h. (iv) Aliquots of 0.25 mg biomass hydrolysates are incubated at 40°C with an N₂ flow to decrease the time of the water evaporation process. This step is essential because the derivatizing agent is sensitive to moisture. (v) N,N-dimethylformamide (DMF) (50 µL) is added to each of the aliquots. (vi) N-methyl-N-(*tert*-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) (50 µL) is added to each sample, and then the samples are heated at 70°C for 30 min. (vii) Two split ratios (1:50 and 1:100), duplicate samples per split ratio, and 3–5 injections per sample are analyzed per each experimental point. We recommend that a different sample be prepared for each split ratio. It was observed that the samples were degrading, if left still long after derivatization.

The amino acid mixture is separated

by GC using a temperature program that starts at 140°C and increases up to 250°C at a rate of 3°C per min. The total time of a GC-MS run is 40 min. Based on the described protocol, the smallest sample volume to be withdrawn from the reactor is equal to $[0.5 \times (\text{biomass concentration in the reactor})^{-1}] \text{ mL}$. However, there is potential for a 10× decrease if needed, barring any additional experimental error due to the processing of much smaller quantities, since each biomass aliquot is currently diluted in 100 µL DMF and MTBSTFA combined to obtain a concentration of 2.5 mg/mL, while only 3–5 injections of 1 µL are needed for each sample [i.e., 10 µL at a maximum (in the absence of duplicates)]. This means, for example, that for a biomass concentration of 2 g/L in the reactor, only 25 µL of sample needs to be withdrawn.

This protocol was applied for the measurement of the mass isotopomer

distribution of 13 amino acids and 2–4 fragments per amino acid in the biomass of a *C. glutamicum* continuous culture grown at 0.1 h^{-1} under glucose limitation, after the introduction of 50% $[1-^{13}\text{C}]$ glucose (11). Table 1 shows eight of the measured amino acids. The values shown correspond to the average of three time points at metabolic and isotopic steady-state conditions, duplicate samples per time point, and three injections per sample (i.e., 18 measurements). The relative variance among the 18 measurements was less than or equal to 1% for the most abundant peaks. However, the relative variance of the least abundant peaks varied from 20% to 200%. Because of the high degree of uncertainty associated with these measurements, we recommend that they not be considered in flux quantification analysis because they can seriously compromise the flux estimates (11).

A clear advantage of the ion-trap compared to the quadrupole mass spectrometer is that, due to its configuration, it is possible for the full spectrum of all observable amino acids in biomass hydrolysates to be accurately measured in a single run. While full mass spectra can also be obtained from quadrupole mass spectrometers running in total ion chromatograph (TIC) mode, exact quadrupole MS analysis requires single ion monitoring (SIM). In this mode, 20–25 ions are simultaneously monitored per run. In reality, after taking into consideration the concentration differences of the various amino acids in the biomass to avoid saturation phenomena effects in the measurements, two to three samples per experimental point at different split ratios and 3–5 injections per sample are needed in the ion-trap mass spectrometer to obtain all measurements at the highest degree of precision and accuracy.

cy (in the absence of duplicates). In the case of *C. glutamicum*, 6 μ L of derivatized biomass hydrolysates (the corresponding dry biomass weight can be estimated as described earlier) and 240 min per duplicate sample are adequate to analyze the mass isotopomer distribution of the biomass hydrolysates at each experimental point, when ion-trap mass spectrometer is used. Both the sample volume and the running time are expected to increase if quadrupole mass spectrometer is used and the same degree of accuracy and precision is required (i.e., SIM mode).

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