Identification and verification of derivatized *D*-glucose fragments by mass spectrometry toward metabolic flux analysis (Original research)

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INTRODUCTION

Fluxome analysis or metabolic flux analysis is an *in vivo* quantitative analysis of carbon trafficking in metabolic pathways (Wittmann *et al*., 1998, 2007) and has been widely used to investigate existing and unknown pathways (Schwender, 2008). Metabolic pathways are sequential, enzyme-catalyzed chemical reactions in living cells. These pathways are usually interconnected such that the product of one reaction is the reactant for multiple subsequent reactions (Voet and Voet, 2004; Nelson and Cox, 2008). The enzymes in these phenomenal pathways are capable of accelerating chemistry to a level that can not be achieved synthetically. Even the simplest of organisms like bacteria exhibit high levels of interconnection and sophistication in their pathways (Alberts *et al.*, 2008). Understanding the complexity of these pathways will allow researchers to gather information about the chemical reactions catalyzed at each step of the pathway. Metabolic pathways are involved in important cellular processes including the regulation of substrate concentrations, conversion of non-usable resources to a usable form, recycling metabolites and other intermediates, and transportation and storage of important resources (Lodish *et al.*, 2008). Research in metabolic flux has provided valuable insights to metabolism and systems biology (Wiechert, 2001; Blank and

Kuepfer, 2009). By understanding the trafficking of molecules in a pathway, metabolic

engineers can identify the bottlenecks in a target pathway and improve the efficiency of

that pathway (Roscher *et al.*, 1999). Successful applications include energy conversion in

plants (Libourel and Shachar-Hill, 2008) or enhancing the production of compounds such

as therapeutic drugs in microorganisms (Ro *et al.*, 2006).

An approach to metabolic flux analysis is to apply single or multiple labeled

molecules as a tracer substrate. A labeled molecule is one wherein one of the elements

(in most cases, carbon) is present as multiple isotopes in unnatural proportions. Metabolic

flux analysis mainly involves the stable carbon isotope ^{13}C because the other possibility,

^{14}C, is a radioactive isotope. Molecules that have the same molecular formula but

different masses are called mass isotopomers (Wittmann *et al.*, 2007). Positional

isotopomers are molecules with the same molecular mass and formula, but the position of

the isotopes is different. For example, $[1\text{-}^{13}C]$, $[2\text{-}^{13}C]$, and $[3\text{-}^{13}C]$ pyruvate (a three-

carbon molecule with the formula $C_3H_4O_3$) are positional isotopomers since different

carbon position is labeled. On the other hand, unlabeled pyruvate (mass *m*) and ^{13}C

labeled pyruvate (mass *m + n*) are mass isotopomers, where *n = (# of labeled carbons)* x

(*mass difference of the isotopes*). Since the mass difference between ^{13}C and ^{12}C is 1

atomic mass unit, n is equivalently the number of ^{13}C in the molecule. The mass

isotopomers only describe the number of ^{13}C atoms in the molecule and not the positions

of the labeled carbons. Hence, the distribution of the positional isotopomers determines

the mass isotopomer distribution, while the distribution of mass isotopomers only gives

certain constraints on the distribution of positional isotopomers (Christensen and Nielsen,

1999; Wittmann, 2002). Using both positional and mass isotopomer distributions, the

flux values can be determined using mathematical modeling and computation (Wiechert, 2007; Wiechert *et al.*, 2001, Antoniewicz *et al.*, 2007, Sriram *et al.*, 2007). Because the mass isotopomer distribution is dependent on the mass of the molecule, isotopes that occurs naturally (^{13}C, ^{18}O, ^{2}H, ^{29}Si) may significantly impact the flux analysis. Knowing the percent abundance of these isotopes, a multiple linear regression method may be utilized to correct the mass isotopomer distribution for these naturally abundant isotopes.

One way to measure the isotopomer distribution is by gas chromatography-mass spectrometry (GC-MS) (Wittmann, 2002). The sensitivity, robustness, and small amounts of sample required make GC-MS a powerful analytical method for this purpose. Gas chromatography has a high ability to separate multiple compounds based on physical properties, while mass spectrometry produces fragmentation patterns that are unique for individual compounds based on chemical properties (Dass, 2007; Skoog *et al.*, 2006). The mass spectrum provides ion clusters ($m + 0$, $m + 1$, $m + 2$, … , $m + n$) for each fragment, as well as the abundance or relative intensity of each peak in the ion cluster. By obtaining the abundance of peaks in each ion cluster, a partial or complete labeling pattern can be assembled, which determines the mass isotopomer distribution (Wittmann and Heinzle, 1998). To achieve this, it is necessary to identify the fragment that produced the signal in each individual ion cluster of mass spectrum, and, in particular, how many and which carbons of the molecule composed of the fragment observed. This is the primary motivation leading to this study.

Glucose is a six-carbon molecule that is used by cells from many organisms as an energy source (Nelson and Cox, 2008). Glucose is particularly of interest to metabolic flux analysis because it is the starting point of many metabolic pathways (Alberts *et al.*,

2008). Glycolysis is the process in which a molecule of glucose is converted to two

molecules of pyruvate with the net production of two molecules of adenosine

triphosphate (ATP). Pyruvate is an important metabolite that is involved in multiple

reactions including: decarboxylation to acetyl CoA, carboxylation to oxaloacetate

(OAA), transamination to alanine, and reduction to lactic acid (Voet and Voet, 2004).

Therefore, understanding the fluxes in glucose-metabolizing pathways will provide key

insights to the flux of downstream metabolites in the tricarboxylic acid cycle (TCA

cycle), fatty acid synthesis, and amino acid synthesis. Identification of glucose by GC-

MS requires chemical derivatization since glucose is not volatile. DeJongh *et al*. (1969)

utilized trimethylsilyl (TMS) ethers to derivatize glucose and determined the

fragmentation structures produced by the GC-MS. This study aimed to verify and

determine the carbons in the fragments mentioned in DeJongh *et al*. by using seven types

of labeled glucose: [U-^{13}C], [1-^{13}C], [2-^{13}C], [3-^{13}C], [4-^{13}C], [5-^{13}C], and [6-^{13}C]

glucose. In this study, we reexamined and updated the work of DeJongh *et al.* since mass

spectrometry today is more developed than that of the time of their study. Knowing the

carbons that are in each fragment is essential for determining the distribution of mass

isotopomers and metabolic flux.

EXPERIMENTALS

Materials and GC-MS Parameters.

All the glucose samples were derivatized by adding 1-(trimethylsilyl)-imidazole

in pyridine 1:4 (v/v) (Kitson *et al*., 1996). The following compounds were purchased

from Cambridge Isotope Laboratories (Andover, MA): unlabeled D-glucose, [1-^{13}C]

labeled D-glucose, [U-^{13}C] labeled D-glucose, and [6-^{13}C] labeled D-glucose. Other varieties of labeled D-glucose ([2-^{13}C], [3-^{13}C], [4-^{13}C], and [5-^{13}C]), were supplied by Dr. Jacqueline V. Shanks' laboratory at Iowa State University. The derivatizing agent, 1-(trimethylsilyl) imidazole in pyridine 1:4 (v/v), was purchased form Thermo Fischer Scientific (Waltham, MA). Samples were heated at 60 °C for 30 minutes to complete derivatization. Samples were analyzed by GC-MS (Agilent, Santa Clara, CA): Varian 450GC-300 MS with electron ionization (EI) and quadrupole mass filters. The dimension of the GC column was 30m x 0.25mm. The injection volume was 1 µL, and the injection temperature was 200 °C. The carrier gas (helium) flow was 1.5 mL/min. The oven temperature was held at 180 °C for 1 minute, and was raised to 200 °C at a rate of 15 °C/min. The oven temperature was then increased to 250 °C at 20 °C/min, and was held at 250 °C for 1 minute. The mass spectrometer was in full scan mode at ionizing energy of 70 eV. Mass distributions were corrected for natural abundance by a program using MATLAB (Mathworks Co., Natick, MA).

Analysis of the relative intensities.

The unlabeled glucose spectrum contained 15 fragments. The goal of the study was to determine how many and which carbons of glucose are represented in these fragments. As mentioned previously, this information is essential for assembling a mass isotopomer distribution, which in turn will provide information about metabolic flux. Each labeled glucose spectrum was compared to the unlabeled glucose spectrum to determine which carbons of glucose were in each fragment observed. For instance, Figure 1 shows a fragment with a m/z of 204 on the spectrum of unlabeled glucose.

Compared to this, [U-^{13}C] labeled glucose produced a fragment with m/z of 206, which is shifted upwards by 2 mass units. Since only the carbons on glucose were labeled with ^{13}C, any mass differences observed indicate that the labeled carbon(s) is in the fragment. Together these observations imply that the fragment of m/z 204 contains two carbons of glucose. Similarly, the spectra of other singly labeled glucose can be compared to the spectrum of the unlabeled glucose. Both [2-^{13}C] and [3-^{13}C] produced a fragment of m/z 205, which are +1 shifts from the m/z 204 of unlabeled glucose. Since only one carbon was labeled with ^{13}C, the 204 fragment contains both the second and third carbon of glucose. For [1-^{13}C] labeled glucose, a peak of m/z 204 was observed and there was no increase in mass. This was also seen for the spectra of [4-^{13}C], [5-^{13}C], and [6-^{13}C] labeled glucose. Consolidation of the information from the spectra of all types of labeled/unlabeled glucose, leads to the conclusion that the m/z 204 fragment contains two carbons of glucose, corresponding to the carbons C-2 and C-3 (second and third carbons, respectively) of glucose. Given a closer look at Figure 1, it can be seen that m/z 191 contains the first carbon of glucose. All 15 fragments of glucose were treated with this analysis and are summarized in Table 1.
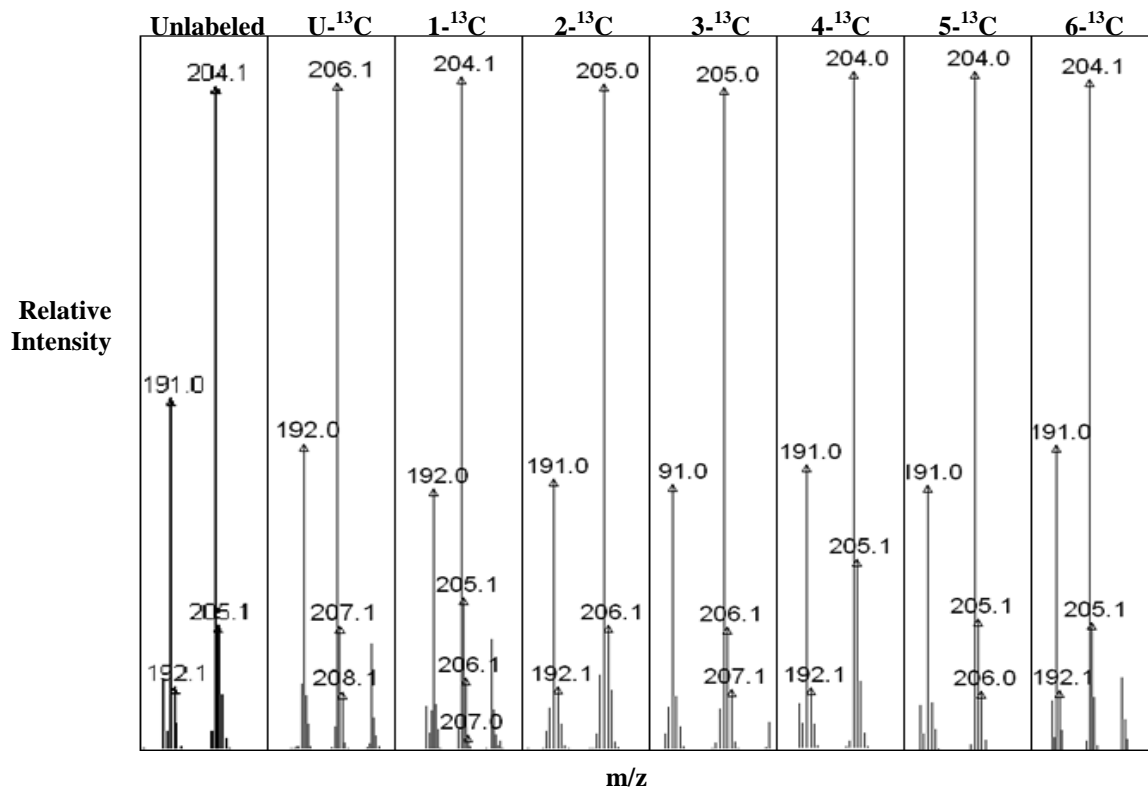
Figure 1. Comparison of relative intensities across different glucose samples. For m/z 204, U-^{13}C displayed a mass increase by 2 indicating the fragment contains two carbons of glucose. Both 2-^{13}C and 3-^{13}C had a mass increase by 1, and all other labels did not display a mass increase. This analysis suggests that the m/z 204 fragment contains the second and third carbon of glucose. This analysis was performed to other peaks, and the full spectra can be found in a separate document.

RESULTS AND DISCUSSION

Separation of molecules in chromatogram.

The gas chromatography parameters were determined to optimize resolution and time. Two large peaks and one small peak were observed on the gas chromatography. The small peak has the lowest retention time (t_R = 3.325) which corresponds to the five-member-ring isomer form of glucose (Catoire *et al.*, 2010). The peak is composed of 1,2,3,5,6-pentakis-O-(trimethylsilyl)-α-D-galactofuranose and 1,2,3,5,6-pentakis-O-(trimethylsilyl)-β -D-galactofuranose, but the peak was not resolved. The two larger peaks, t_R = 3.625 and t_R = 4.250, corresponded to the two six-member-ring isomer forms,

1,2,3,4,6-pentakis-O-(trimethylsilyl)-α-D-glucopyranose and 1,2,3,4,6-pentakis-O-(trimethylsilyl)-β-D-glucopyranose, respectively.

Identification of novel fragments and discrepancy from DeJongh et al.

Peaks that appeared beyond m/z 400 had intensities that were too low for analysis. Both the 1,2,3,4,6-pentakis-O-(trimethylsilyl)-α-D-glucopyranose and 1,2,3,4,6-pentakis-O-(trimethylsilyl)-β-D-glucopyranose isomers gave nearly identical mass spectra. Unlabeled glucose exhibited mass peaks at m/z = 73, 89, 117, 129, 146, 189, 191, 204, 217, 231, 243, 291, 305, 345, and 393.   Using uniformly labeled glucose and singly labeled glucose at different carbon positions, the fragments corresponding to each peak were investigated.

It was observed that m/z 73, m/z 133, and m/z 147 were peaks produced by the trimethylsilyl derivatizing agent, since these peaks did not shift for any of the ^{13}C-labeled glucose used (Table 1). Two pairs of peaks were too close together for analysis, m/z 189, 191 and m/z 146, 147. Comparing to the structures proposed by DeJongh *et al.*, there was a discrepancy for m/z 129. The mass intensities of [2-^{13}C] and [5-^{13}C] labeled glucose showed that m/z 129 contained fragments of either [2 3 4] or [3 4 5] instead of the proposed [4 5 6]. Furthermore, the mass intensities of [1-^{13}C] and [4-^{13}C] labeled glucose showed that the m/z 291 and m/z 305 both might each be composed of two fragments, [1 2 3] and/or [2 3 4]. Interestingly, m/z 231, 243, 345, 393 were peaks that were not mentioned by DeJongh *et al*. It was found that m/z 231 is a [3 4 5 6] fragment, m/z 243 is a [2 3 4 5 6] fragment, m/z 345 is a [1 2 3 4 5 6] fragment, and m/z 393 is a

[1 2 3 4] fragment. Several mixtures of labeled glucose were analyzed to determine whether m/z 129, 291, 305 contain more than one fragments.

Table 1. Summary of fragmentation results after applying the analysis shown in Figure 1. Each number corresponds to the mass peak in each spectrum for every m/z peaks of interest. Four novel peaks were identified (m/z 231, 243, 345, 393). Two peaks (m/z 146, 189) were not analyzed due to overlapping mass distributions with adjacent peaks. Three peaks (m/z 129, 204, 291) disagreed with the fragments proposed by DeJongh et al. Further information provided in separate document. Rows with red text represent new or updated information found in this study. For m/z 129, 291, and 305, the analysis did not yield a clear fragment, thus mixtures were made to further identify distinguish the correct fragment

m/z	Unlabeled	U-13C	# of Carbons	Mass of fragment in spectrum of ^{13}C labeled glucose						DeJongh et al. fragment	Experimental fragment
				1-^{13}C	2-^{13}C	3-13C	4-13C	5-13C	6-13C		
89	89	90	1	89	89	89	89	89	90	[6]	[6]
117	117	119	2	117	117	117	117	118	118	[5 6]	[5 6]
129	129	132	3	129	130	130	130	130	129	[4 5 6]	[2 3 4] + [3 4 5]?
Overlap with TMS peak at m/z 133											
146	146	Overlap with TMS peak at m/z 147								[1 2]	
189	189	192	3	Overlap with m/z 191							
191	191	192	1	192	191	191	191	191	191	[1]	[1]
204	204	206	2	204	205	205	204	204	204	[2 3] or [3 4]	[2 3]
217	217	220	3	217	218	218	218	217	217	[2 3 4]	[2 3 4]
231	231	235	4	231	231	232	232	232	232	not identified	[3 4 5 6]
243	243	248	5	243	244	244	244	244	244	not identified	[2 3 4 5 6]
291	291	294	3	291/292	292	292	291/292	291	291	[2 3 4]	[1 2 3] or [2 3 4]?
305	305	308	3	305/306	306	306	305/306	305	305	[2 3 4]	[1 2 3] or [2 3 4]?
345	345	351	6	246	246	246	246	246	246	not identified	[1 2 3 4 5 6]
393	393	397	4	394	394	394	394	393	393	not identified	[1 2 3 4]

Using different mixtures to resolve single and multiple fragments.

Since the analysis mentioned above did not indicate a distinct fragment for m/z 129, 291, and 305, there may be a possibility that the two fragments of the same mass may contribute to the m/z peaks observed. Thus, several mixtures containing two different singly labeled glucose was made. It was expected that the relative intensity of a peak containing one fragment would display dependency on the concentration, while a peak containing more than one fragment would not be. Monitoring the changes in peak intensities in relation to changes in compositions will allow us to determine whether the peak contains more than one fragment. Three compositions of 50:50, 25:75, and 75:25

were made containing [2-^{13}C] and [5-^{13}C] singly labeled glucose. Similarly, [1-^{13}C] and

[4-^{13}C] labeled glucose mixtures were made. A pre-written MATLAB script (Sriram *et*

al., 2008; van Winden *et al.*, 2002) was used to correct for naturally abundant isotopes of

elements other than metabolic carbon, which can be introduced through the derivatizing

agent.   For m/z 291 and m/z 305, the [1-^{13}C] and [4-^{13}C] mixtures were analyzed (Table

2). The $m + 0$ peak decreased as [4-^{13}C] decreased, and the $m + 2$ and $m + 3$ peaks

increased with increasing [1-^{13}C]. The $m + 0$ peak is dependent on the [4-^{13}C], while the

$m + 1$ peak is dependent on [1-^{13}C]. This seems to indicate the m/z 291 fragment is [1 2

3] and not [2 3 4]. Strangely, the $m + 2$ and $m + 3$ peaks did not give any clear trend.

For m/z 305, it was observed that the $m + 0$ peak is dependent on the amount of [1-^{13}C],

while the $m + 1$ and $m + 2$ peaks are dependent on [4-^{13}C]. This suggest that the m/z 305

peak contains the fragment [2 3 4] and not [1 2 3]. Similar to m/z 291, the $m + 3$ peak

did not give any clear trend. To determine if m/z 129 contains [2 3 4] or [3 4 5], the [2-

^{13}C] and [5-^{13}C] mixtures was analyzed. No significant trend was observed for $m + 0$, m

$+ 1$, $m + 2$, or $m + 3$ peaks. This suggests that the m/z 129 fragment may contain both [2

3 4] and [3 4 5].

Table 2. Relative intensities for label mixtures corrected for natural abundance. Spectra are not shown. For m/z 291, the relative intensity for m+0 decreased with increasing [1-^{13}C] composition, while m+1 increased indicating a [1 2 3] fragment. The opposite pattern was observed for m/z 305, indicating a [2 3 4] fragment. With m/z 129, no clear trend was observed, which possibly contains both the [2 3 4] and [3 4 5] fragments.

m/z		1-^{13}C+4-^{13}C (25:75)	1-^{13}C+4-^{13}C (50:50)	1-^{13}C+4-^{13}C (75:25)	m/z		2-^{13}C+5-^{13}C (25:75)	2-^{13}C+5-^{13}C (25:75 trial2)	2-^{13}C+5-^{13}C (50:50)	2-^{13}C+5-^{13}C (75:25 trial2)
291	*m+0*	0.4979	0.4698	0.4499	129	*m+0*	0.2868	0.2850	0.3021	0.2565
	m+1	0.4753	0.5007	0.5137		*m+1*	0.4914	0.4921	0.4807	0.4425
	M+2	0.0258	0.0213	0.0303		*m+2*	0.1599	0.1623	0.1638	0.2543
	m+3	0.0008	0.0080	0.0059		*m+3*	0.0618	0.0604	0.0533	0.0466
305	*m+0*	0.3131	0.3762	0.4785						
	m+1	0.6180	0.5690	0.4659						
	m+2	0.0563	0.0462	0.0386						
	m+3	0.0124	0.0084	0.0168						

CONCLUSION

   Correctly identifying the fragments in each ion clusters is critical to the assembly of a mass isotopomer distribution. As a consequence, an inaccurate mass isotopomer distribution can significantly impact the result of metabolic flux analysis. Due to the advances made in mass spectrometry within the past decades, this study aimed to update the work done by DeJongh *et al.* (1969) and validate those fragments proposed. Using several types of ^{13}C labeled glucose, this study was successful in verifying many of the fragments identified by DeJongh *et al*. and elucidated the discrepancies in three of these fragments (m/z 129, 204, 291). Furthermore, four novel fragments were detected (m/z 231, 243, 345, 393). The experiment with different label mixtures was able to distinguish whether a mass peak contained one or more fragments. All six carbons in glucose that partitioned into fragments can be monitored by the fragments identified in this study. Metabolic flux analysis using GC-MS have been applied to microorganisms including *E. coli* (Fischer and Sauer, 2003), *C. glutamicum* (Kiefer *et al.,* 2004), *Bacillus subtilis* (Fuhrer *et al.,* 2005), and *Penicillium chrysogenum* (Thykaer *et al.*, 2002) to understand metabolism and metabolite productions. Experiments using isotopic labels and GC-MS have also been utilized in the biomedical field to humans and animals relating metabolism to diseases (Des Rosiers *et al.*, 2004; Kelleher, 2001). The novelty of the result found in this study contributes to metabolic flux analysis and many different fields of research. By correctly identifying the fragments of glucose, more accurate information about mass isotopomer distribution and metabolic flux can be gathered.

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