Anal. Chem. **2008,** *80,* 9508–9516

Simultaneous Quantification of Metabolites Involved in Central Carbon and Energy Metabolism Using Reversed-Phase Liquid Chromatography−Mass Spectrometry and in Vitro ^{13}C Labeling

Wen-Chu Yang,[†] Miroslav Sedlak,[‡,§] Fred E. Regnier,[||] Nathan Mosier,[‡,§] Nancy Ho,[‡,§] and Jiri Adamec*[,†]

Bindley Bioscience Center, Laboratory of Renewable Resources Engineering, Department of Agricultural and Biological Engineering, and Department of Chemistry, Purdue University, West Lafayette, Indiana 47907

Comprehensive analysis of intracellular metabolites is a critical component of elucidating cellular processes. Although the resolution and flexibility of reversed-phase liquid chromatography−mass spectrometry (RPLC−MS) makes it one of the most powerful analytical tools for metabolite analysis, the structural diversity of even the simplest metabolome provides a formidable analytical challenge. Here we describe a robust RPLC−MS method for identification and quantification of a diverse group of metabolites ranging from sugars, phosphosugars, and carboxylic acids to phosphocarboxylics acids, nucleotides, and coenzymes. This method is based on in vitro derivatization with a ^{13}C-labeled tag that allows internal standard based quantification and enables separation of structural isomer pairs like glucose 6-phosphate and fructose 6-phosphate in a single chromatographic run. Calibration curves for individual metabolites showed linearity ranging over more than 2 orders of magnitude with correlation coefficients of $R^2 > 0.9975$. The detection limits at a signal-to-noise ratio of 3 were below 1.0 μM (20 pmol) for most compounds. Thirty common metabolites involved in glycolysis, the pentose phosphate pathway, and tricarboxylic acid cycle were identified and quantified from yeast lysate with a relative standard deviation of less than 10%.

The metabolome is uniquely different than the proteome, transcriptome, and genome in not being directly encoded by the genome. Also, the number of metabolites in a metabolome is not related to the number of proteins or genes in a cell. This is because metabolites are formed and transformed by proteins, but necessarily on a stoichiometric basis. Metabolites can also play a role in their own formation along with the synthesis of other metabolites by impacting the function of proteins directly. This occurs by direct interaction with proteins, or indirectly through changes in physical−chemical conditions inside the cell such as in lowering levels of ATP or changing cellular pH. Moreover, low molecular weight molecules are important components in the communication network among cells, tissues, and even between organisms as in the case of pheromones. A critical component of these phenomena is that most of them are dependent on metabolite concentration. This means that quantification of metabolites is of major importance in elucidating the regulatory impact of metabolites in biological systems.[1]

Although enzyme-based assays for individually determining certain metabolites have been available for some time,[2−4] these assays are time-consuming and limited to small number of metabolites, depending on the availability of the enzymes. Simultaneous quantification of multiple metabolites is highly preferred for comprehensive study of cellular metabolism. Capillary electrophoresis−mass spectrometry (CE−MS) is a promising tool for ionic metabolites analysis,[5,6] but generally robustness and sensitivity need to be improved.[7] Currently, liquid chromatography (LC) is a predominant technique for these studies. Due to metabolites anionic property, anion-exchange chromatography (AEC) with UV detection was first used for the analysis of nucleotides.[8] When the use of AEC was extended to sugar phosphates or carboxylic acids, other detection techniques, such as pulsed amperometric detection,[9−11] potentiometric detection,[12]

* To whom correspondence should be addressed. Phone: 765-496-6148. Fax: 765-496-1518. E-mail: jadamec@purdue.edu.

[†] Bindley Bioscience Center.

[‡] Laboratory of Renewable Resources Engineering.

[§] Department of Agricultural and Biological Engineering.

[||] Department of Chemistry.

(1) Fiehn, O.; Kopka, J.; Trethewey, R. N.; Willmitzer, L. *Anal. Chem.* **2000**, *72*, 3573–3580.

(2) Hajjaj, H.; Blanc, P. J.; Goma, G.; Francois, J. *FEMS Microbiol. Lett.* **1998**, *164*, 195–200.

(3) Ruijter, G. J. G.; Visser, J. *J. Microbiol. Methods* **1996**, *25*, 295–302.

(4) Theobald, U.; Mailinger, W.; Bates, M.; Rizzi, M.; Reuss, M. *Biotechnol. Bioeng.* **1997**, *55*, 305–316.

(5) Soga, T.; Ueno, Y.; Naraoka, H.; Ohashi, Y.; Tomita, M.; Nishioka, T. *Anal. Chem.* **2002**, *74*, 2233–2239.

(6) Toya, Y.; Ishii, N.; Hirasawa, T.; Naba, M.; Hirai, K.; Sugawara, K.; Igarashi, S.; Shimizu, K.; Tomita, M.; Soga, T. *J. Chromatogr., A* **2007**, *1159*, 134–141.

(7) Cai, J. Y.; Henion, J. *J. Chromatogr., A* **1995**, *703*, 667–692.

(8) Cohn, W. E. *Science* **1949**, *109*, 377–378.

(9) Groussac, E.; Ortiz, M.; Francois, J. *Enzyme Microb. Technol.* **2000**, *26*, 715–723.

(10) Jensen, N. B.; Jokumsen, K. V.; Villadsen, J. *Biotechnol. Bioeng.* **1999**, *63*, 356–362.

(11) Smits, H. P.; Cohen, A.; Buttler, T.; Nielsen, J.; Olsson, L. *Anal. Biochem.* **1998**, *261*, 36–42.

10.1021/ac801693c CCC: $40.75
Published on Web 11/14/2008

or conductometric detection[9,13−16] were used to circumvent metabolites insufficient UV absorbance and, consequently, low UV detection sensitivity.

One of the most common ways of analyzing metabolites is through separation by gas or liquid chromatography followed by identification and quantification through mass spectrometry (MS).[17,18] Signal intensity of an analyte in MS depends on its concentration and ionization efficiency. Ionization efficiency not only varies between analytes but can depend on other components in the matrix, particularly in the case of electrospray ionization as used in LC−MS. This problem can be circumvented in LC−MS quantification through the use of a ^{13}C-coded internal standard that coelutes with the analyte and has an ionization environment identical to the analyte. Synthesizing the requisite ^{13}C-coded internal standard is generally simple when the number of analytes being determined is small. When large, the requisite number of syntheses can become prohibitive. Although it is possible to biosynthesize ^{13}C-coded metabolites,[19] a comprehensive collection of internal standard metabolites is generally not available. Some have used standard addition methods for MS quantification[19−22] to circumvent this problem. However, the MS response can change over time due to changes in the MS instrument.[23]

The paper reports a new postbiosynthetic (in vitro) stable isotope encoding procedure called group specific internal standard technology (GSIST).[24,25] In GSIST, metabolites from control samples (or metabolite standards) and experimental samples are derivatized with chemically identical but isotopically distinct labeling agents. In effect, sample components are chemically coded according to their sample origin. After mixing these derivatized metabolites, each molecule from the control or standard sample serves as an internal standard for determining the concentration of the corresponding compounds in the experimental sample. Recent studies in our laboratory have focused on derivatizing agents targeting primary amines[10] and carboxyl groups.[24] Although these coding agents work well for specific classes of molecules, they have some limitations in global and pathway-targeted approaches since not all molecules contain the same functional groups. For this reason we have introduced a new derivatization reagent targeting multiple functional groups that is more suitable for quantification of specific metabolic pathways as well as differential global metabolomics. An in vitro aniline derivatization is used that allows for absolute quantification of known compounds or relative quantification of unknown compounds without requiring standards. Moreover large numbers of analytes can be analyzed in a single LC−MS run.

The utility of in vitro aniline derivatization was applied to the quantification of intermediates in central carbon and energy metabolism. Among the whole cellular metabolic network, central carbon metabolism, composed of glycolysis, the pentose phosphate pathway, and the tricarboxylic acid cycle (TCA), plays a key function in substrate degradation, energy and cofactor regeneration, and biosynthetical precursor supply. There are more than 35 intermediates that belong to several categories of chemical compounds: phosphorylated sugars, phosphocarboxylic acids, carboxylic acids, nucleotides, and cofactors. Simultaneous analysis of these compounds is a challenging analytical problem. This paper describes a new in vitro $^{13}C_6$ labeling method that allows accurate determination of most intermediates involved in central carbon and energy metabolism in a single 30 min reversed-phase liquid chromatography−mass spectrometry (RPLC−MS) run.

EXPERIMENTAL SECTION

Materials and Reagents. All metabolite standards, aniline, aniline-$^{13}C_6$, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), tributylamine (TBA), triethylamine (TEA), and HPLC grade water were purchased from Sigma-Aldrich (St. Louis, MO). HPLC grade acetonitrile (ACN) was obtained from Mallinckrodt Baker (Phillipsburg, NJ).

Yeast Growth and Fermentation. *S. cerevisiae* (ATCC 4124) was inoculated directly from the agar plates into 5 mL of YEP + 2% glucose medium. The cultures were incubated in a shaker at 30 °C and 200 rpm and grown aerobically overnight. The following morning, the culture were transferred directly to 100 mL YEP + 2% glucose in a 300 mL Erlenmeyer flask equipped with a side arm (Bellco), which allows for direct monitoring of the growth of yeast cultures by a Klett colorimeter (Manostat Corp.). The cultures were incubated as described above until cell density reached 500 KU. At this point, 24 mL of (50%) glucose was added to the flask. The flask was then sealed with Saran wrap to allow fermentation to proceed under largely anaerobic conditions. The cultures were incubated as described above, with cell growth monitoring by Klett colorimeter. One milliliter samples of the mixture were removed at proscribed intervals to monitor fermentation. The sample for intracellular metabolite analysis was taken 3 h after fermentation started. Glucose and fermentation products such as glycerol, acetic acid, and ethanol were analyzed by high-performance liquid chromatography (HPLC) using HPX 87H (8 mm × 300 mm, Bio-Rad Laboratories, CA).

Sample Preparation. Sampling was performed as described by Gonzales et al.[26] and Lange et al.[27] Briefly, 5 mL of yeast culture was sprayed into 50 mL centrifugation tubes (Oak Ridge centrifugation tube, FEP) containing 26 mL of cold solution with 60% (v/v) analytical grade methanol (Mallinckrodt), kept at −45 °C

(12) Picioreanu, S.; Poels, I.; Frank, J.; van Dam, J. C.; van Dedem, G. W.; Nagels, L. J. *Anal. Chem.* **2000**, *72*, 2029–2034.
(13) Bhattacharya, M.; Fuhrman, L.; Ingram, A.; Nickerson, K. W.; Conway, T. *Anal. Biochem.* **1995**, *232*, 98–106.
(14) Hull, S. R.; Montgomery, R. *Anal. Biochem.* **1994**, *222*, 49–54.
(15) Ritter, J. B.; Genzel, Y.; Reichl, U. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2006**, *843*, 216–226.
(16) Vogt, A. M.; Ackermann, C.; Noe, T.; Jensen, D.; Kubler, W. *Biochem. Biophys. Res. Commun.* **1998**, *248*, 527–532.
(17) Stephanopoulos, G.; Alper, H.; Moxley, J. *Nat. Biotechnol.* **2004**, *22*, 1261–1267.
(18) Wamelink, M. M.; Struys, E. A.; Huck, J. H.; Roos, B.; van der Knaap, M. S.; Jakobs, C.; Verhoeven, N. M. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2005**, *823*, 18–25.
(19) Huck, J. H.; Struys, E. A.; Verhoeven, N. M.; Jakobs, C.; van der Knaap, M. S. *Clin. Chem.* **2003**, *49*, 1375–1380.
(20) Buchholz, A.; Takors, R.; Wandrey, C. *Anal. Biochem.* **2001**, *295*, 129–137.
(21) Luo, B.; Groenke, K.; Takors, R.; Wandrey, C.; Oldiges, M. *J. Chromatogr., A* **2007**, *1147*, 153–164.
(22) van Dam, J. C.; Eman, M. R.; Frank, J.; Lange, H. C.; van Dedem, G. W. K.; Heijnen, S. J. *Anal. Chim. Acta* **2002**, *460*, 209–218.
(23) Coulier, L.; Bas, R.; Jaspersen, S.; Verheij, E.; van der Werf, M. J.; Hankemeier, T. *Anal. Chem.* **2006**, *78*, 6573–6582.
(24) Yang, W. C.; Adamec, J.; Regnier, F. E. *Anal. Chem.* **2007**, *79*, 5150–5157.
(25) Yang, W. C.; Mirzaei, H.; Liu, X. P.; Regnier, F. E. *Anal. Chem.* **2006**, *78*, 4702–4708.
(26) Gonzalez, B.; Francois, J.; Renaud, M. *Yeast* **1997**, *13*, 1347–1355.
(27) Lange, H. C.; Eman, M.; van Zuijlen, G.; Visser, D.; van Dam, J. C.; Frank, J.; de Mattos, M. J.; Heijnen, J. J. *Biotechnol. Bioeng.* **2001**, *75*, 406–415.

in the cryostat bath (HAAKE Phoenix II P1). After 3 min in −45 °C in the cryostat bath, the mixture was centrifuged at 500*g* for 5 min in a Beckman Avanti J-30I set at −20 °C, the supernatant was discarded, and the cell pellet was resuspended in 5 mL of cold quenching solution (−45 °C) followed by a second identical centrifugation. Tubes containing washed pellets were kept at −45 °C in the cryostat bath. The metabolites were extracted from cell pellets with boiling ethanol. Tubes containing cell pellets were placed into a 90 °C water bath and immediately overlain by a solution of 75% (v/v) boiling 100% ethanol, vortexed, and kept for 3 min in a 90 °C water bath. After 3 min the tubes were placed into a −80 °C freezer.

LC−MS. The HPLC−ESI-MS system consisted of a capillary HPLC system (1100 series LC, Agilent) and an electrospray ionization (ESI) source of time-of-flight (TOF) mass spectrometer (MSD TOF, Agilent). The system was controlled by ChemStation software (Agilent). The autosampler was set at 10 °C. Separations were performed on a Zorbax C8 column (2.1 mm × 150 mm, Agilent). The elution started from 95% mobile phase A (5 mM TBA aqueous solution, adjusted to pH 5.0 with acetic acid) and 5% mobile phase B (100% ACN), raised to 70% B in 25 min, further raised to 100% B in 2 min, and then held at 100% B for 3 min. The flow rate was set at 0.3 mL/min with injection volume as 20.0 μL. The column was preconditioned by pumping the starting mobile phase mixture for 10 min. LC−ESI-MS chromatograms were acquired in negative ion mode under the following conditions: capillary voltage of 4000 V and fragmentor of 165 V, dry temperature at 300 °C, dry gas flow maintained at 8.0 L/min, and an acquisition range of m/z 150−1000.

General Labeling Protocol. A solution of 3.0 M aniline or aniline-$^{13}C_6$ was prepared in water and titrated by 6 M hydrochloric acid to pH 4.5. EDC at 20.0 mg/mL was prepared freshly in water. A 100 μL working or sample solution was added with 10 μL of 3.0 M aniline or aniline-$^{13}C_6$, followed by adding 10 μL of 200.0 mg/mL EDC (∼5 μmol). The mixture was vortexed and then incubated with gentle shaking at ambient temperature (∼22 °C) for 2 h. The labeling reaction was stopped by adding 2 μL of triethylamine.

Method Evaluation and Validation. Stock solutions for each standard were prepared in water at 10 mM. Individual standard metabolite solutions (each 0.1 mM) were separately labeled with aniline and aniline-$^{13}C_6$, and a 1:1 mixture was analyzed. The LC peak and MS spectrum patterns were examined to confirm the labeling reaction and to gain the m/z value for sample analysis. A 14.3 μM standard mixture including 33 analytes was prepared from the stock solutions and used for optimizing the labeling and separation conditions. To determine the detection limit and linearity range, a mixture of 33 analytes at various concentrations for each component (depending on their MS response intensity) was prepared. A series of dilutions from this mixture were labeled with aniline. Another aliquot of this mixture was labeled with aniline-$^{13}C_6$, and a certain amount of this derivative mixture was added into the above series of solutions as references for MS response calibration.

RESULTS AND DISCUSSION

Labeling Strategy. The compounds directly involved in central carbon metabolism contain carbonyl, phosphate, and carboxyl groups. It was our intent to develop a relatively global labeling approach which could introduce ^{13}C-coded hydrophobic moieties into all analytes of interest and allow us to determine all these compounds in a single RPLC−MS run. Reductive amination with amine-containing reagent is a common way to label carbonyl groups.[28] Amine-containing reagents have also been reported to label carboxyl and phosphate groups by nucleophilic addition using a water-soluble carbodiimide such as EDC.[29,30] On the basis of these observations, isoforms of aniline (including aniline-$^{13}C_6$) were selected for experiments in isotope coding based on the reactions seen in Figure 1.

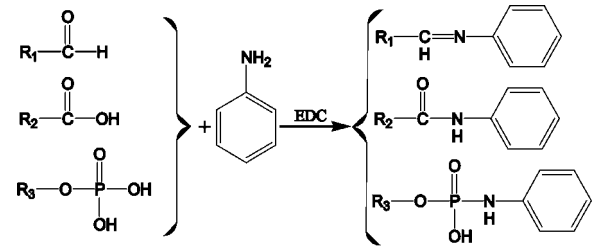

Figure 1. General labeling schemes for carbonyl, phosphoryl, and carboxyl with aniline.

Optimization of Derivatization Reaction. Following optimization of reaction conditions phosphomonoesters were labeled with aniline at 20 °C and pH 4.5−5.5 for 1 h using EDC catalysis.[30] These conditions are similar to those used in primary amine labeling of carboxyl groups using EDC.[29] On the other hand, carbonyl labeling with primary amines is often achieved in a nonaqueous solvent such as methanol with ∼30% acetic acid at roughly 50 °C. Because the intermediate Schiff base formed in carbonyl labeling is unstable under acidic conditions, it was generally reduced with $NaCNBH_3$ to form a stable secondary amine. Carbonyl groups in glucose, xylose, and phosphosugars were also derivatized under these conditions, even without the addition of acetic acid. Inclusion of acid in the reaction possibly converts ketoses to aldoses but was found to bring no advantage here because the Schiff base intermediate is unstable at pH 4.5. In fact, adjusting the pH to 10 by adding 2 μL of TEA at the conclusion of labeling appreciably increased stability. No significant degradation was observed over 3 days when a labeled sample was placed in the autosampler at 10 °C. Addition of $NaCNBH_3$ was also investigated, and the overall LC separation deteriorated with the use of this reagent. Therefore, this reduction step was omitted.

Aniline concentrations from 0.3 to 6 M were used to further optimize the primary labeling conditions above using extracted ion chromatographic peak intensity With increasing concentration, labeling yield increased especially for carbonyl-containing analytes and some carboxylic acids, such as succinic and furmaric acid. Labeling time was examined from 10 to 150 min. The yield was roughly 70% in 10 min and slowly increases to nearly quantitative derivatization at 105 min where most unlabeled analytes were below 0.1%. Raising labeling temperature from ambient to 50 °C decreased labeling, probably owing to acceleration of EDC hydrolysis. The final optimized protocol is to label at ambient

(28) Baxter, E. W.; Reitz, A. B. *Org. React.* **2002**, *59*, 1–714.
(29) Ford, Q. L.; Burns, J. M.; Ferry, J. L. *J. Chromatogr., A* **2007**, *1145*, 241–245.
(30) Ivanovskaya, M. G.; Gottikh, M. B.; Shabarova, Z. A. *Nucleosides Necleotides* **1987**, *6*, 913–934.

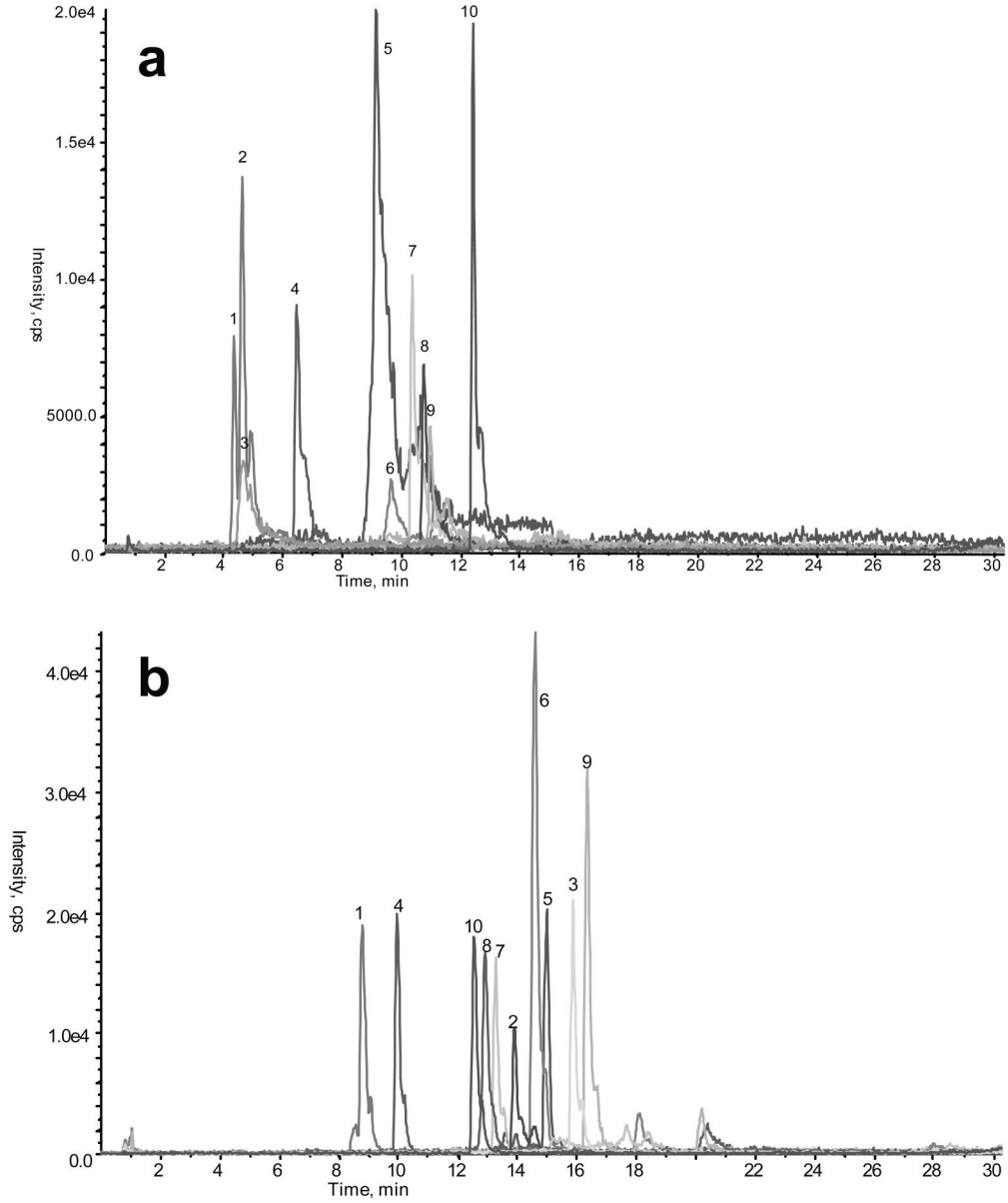

Figure 2. RPLC−MS performance comparison for 12.0 μM free (a) and aniline-labeled (b) metabolites. Metabolites: 1, glucose 6-phosphate; 2, fructose 6-phosphate; 3, D-erythrose 4-phosphate; 4, adenosine 5-monophosphate; 5, malate; 6, ketoglutate; 7, gluconate 6-phosphate; 8, fructose 1,6-bisphosphate; 9, phospho(eno)pyruvate; 10, flavin adenine dinucleotide (reference peak). LC−MS conditions are described in the Experimental Section.

temperature for 2 h with at least a 300-fold excess of aniline at pH 4.5.

Optimization of Analytical Conditions. Labeled standards were analyzed by ion pairing RPLC followed by ESI-MS in the negative ion mode of ionization. TBA was adapted as an ion paring agent because of its promising performance in the separation of unlabeled central carbon metabolites.[21] Optimization of the separation focused on mobile phase pH and TBA concentration. A pH of 5.0 was found to be the optimum for all the analytes. To shorten analysis time, 5 mM TBA was used.

Analytical performance enhancement of several representative labeled metabolites is demonstrated in Figure 2. Note that there is no significant variation from Figure 2, parts a to b, in peak intensity and retention time of reference peak 10. It is concluded that labeling improved the separation in terms of peak shape, resolution, and selectivity, and also enhanced MS sensitivity. The change of separation selectivity is obviously attributed to different labeling patterns of the isomers, such as glucose 6-phosphate and fructose 6-phosphate, which will be discussed further below. The enhancement in peak intensity results from the increase in analyte hydrophobicity after labeling.[25]

Method Evaluation. Most metabolites studied here have more than one functional group which could be labeled. To validate the labeling reaction, standard metabolites were individually labeled with aniline and aniline-$^{13}C_6$. A mixture of equal amounts of the two labeled solutions was analyzed. Labeling patterns can be easily recognized by examining the spectrum. Chromatographic peaks should contain a doublet set of ions, e.g., two major ions of similar peak intensity and a mass difference of $6n$ (n = 1, 2, 3) where n is the number of functional groups in the molecule that were labeled. Also, the m/z of the first ion in the doublet should be the molecular weight of the metabolite plus

Table 1. Labeling and Identification RPLC−MS Results of Standard Metabolites

peak no.	compd	retention time (min)	*m/z* value: ^{12}C labeling	*m/z* value: ^{13}C labeling	*m/z* value: nonlabeling	labeling pattern	MS species
1	glycerol 3-phosphate	4.60			152.99	non	$[M - H_2O - H]^-$
2	xylose	4.96	260.07	266.09		mono	$[M + Cl]^-$
3	NAD	5.01			698.08	non	$[M + Cl]^-$
4	glucose	5.27	290.08	296.10		mono	$[M + Cl - H]^-$
5	fructose 6-phosphate	8.81	334.07	340.09		mono	$[M - H]^-$
6	lactic acid	8.93	164.07	170.09		mono	$[M - H]^-$
7	D-ribulose 5-phosphate	9.37	304.06	310.08		mono	$[M - H]^-$
8	AMP	9.97	421.10	421.12		mono	$[M - H]^-$
9	dihydroxyacetone 1-P	10.07	244.04	250.06		mono	$[M - H]^-$
10	NADP	10.31			724.06	non	$[M - H]^-$
11	D-(−)glycerate 3-P	11.94	242.03	248.06		mono	$[M - H]^-$
12	FAD	12.56			784.15	non	$[M - H]^-$
13	ADP	12.56	501.07	507.03		mono	$[M - H]^-$
14	fructose 1,6-bisphosphate	12.93	396.03	402.05		mono	$[M - H]^-$
15	gluconate 6-phosphate	13.29	425.11	437.15		bi	$[M - H]^-$
16	glucose 6-phosphate	13.90	409.12	421.16		bi	$[M - H]^-$
17	NADH	14.16	633.11	639.13		mono	$[M - \text{nicotinamide} + H_2O - H]^-$
18	ketoglutarate	14.61	295.01	307.14		bi	$[M - H]^-$
19	DL-glyceraldehyde 3-P	14.99	319.09	331.13		bi	$[M - H]^-$
20	malate	15.01	283.11	295.15		bi	$[M - H]^-$
21	ATP	15.14	581.03	587.05		mono	$[M - H]^-$
22	D-ribose 5-phosphate	15.25	379.11	391.15		bi	$[M - H]^-$
23	acetyl Co A	15.69			790.11	non	$[M - H]^-$
24	D-erythrose 4-phosphate	15.88	349.09	361.13		bi	$[M - H]^-$
25	phospho(enol)pyruvate	16.38	317.07	329.11		bi	$[M - H]^-$
26	succinate	16.43	267.12	279.16		bi	$[M - H]^-$
27	NADPH	16.47	695.07	701.09		mono	$[M - \text{nicotinamide} - H]^-$
28	fumarate	17.72	265.09	277.13		bi	$[M - H]^-$
29	glycerate 1,3-bisphosphate	17.99	490.09	508.15		tri	$[M - H]^-$
30	oxalacetate	19.70	280.98	293.02		bi	$[M - H]^-$
31	isocitrate	20.14	398.15	416.21		tri	$[M - H]^-$
32	citrate	21.19	416.16	434.22		tri	$[M - H]^-$
33	*cis*-aconitate	22.35	380.98	399.04		tri	$[M - H_2O - H]^-$

75 amu × n (n = 1, 2, 3). Retention time, labeling pattern, and MS species identified are summarized in Table 1.

Some special cases need to be addressed. Since simple labeled sugars are neutral, their chloride adducts were found by negative ESI-MS. Instead of aniline labeling, the dehydrated glycerol 3-P ion was found. This ion probably arose by intermolecular addition of a hydroxyl group at C-1 that attached the EDC-activated C3-phosphate. Phosphoaldoses such as glucose 6-phosphate and ribose 5-phosphate were bislabeled, whereas phosphoketoses such as fructose 6-phosphate and ribulose 5-phosphate were monolabeled. This illustrates that ketoses do not convert to aldoses in the weak acid media. This labeling pattern benefits the separation of these isomers. They are easily separated without the need to fine-tune the separation. NADH and NADPH were labeled, but with loss of the nicotinamide moiety. It is unclear whether this loss is due to in-source fragmentation or the labeling process itself. On the other hand, oxidized forms of the coenzymes NAD, FAD, and NADP were not labeled, and quantification had to be performed by standard addition. It is presumed this is because of the formation of an intramolecular salt between the quaternary amine on the pyridine ring and the negatively charged phosphate group. In cases where multiple phosphates exists within in a molecule, only one phosphate group was labeled, such as with fructose 1,6 bisphophate, ADP, and ATP. On the basis of sensitivity, this method failed to analyze pyruvate.

Figure 3a shows overlapped extracted ion chromatograms from an equimolar mixture of 33 metabolites in which individual components were present at 14.3 μM. Although some of components coelute, they differ in m/z values and are easily differentiated. Some representative MS spectra with doublet ions are shown in Figure 3b−d, giving further confirmation of labeling patterns. Clearly, subsequent to labeling RPLC−MS discriminated between 33 central carbon intermediates within 30 min.

Method Validation. The method was validated by determination of limit of detection (LOD), limit of quantification (LOQ), linearity range correlation coefficient, and within-assay precision for the 33 analytes by analyzing aniline-labeled standard mixtures of variable concentration spiked with a constant amount of the same aniline-$^{13}C_6$-labeled standards (Table 2). For most compounds, the LOD (S/N = 3) and LOQ (S/N = 10) were established below 1.0 and 2.5 μM, respectively. The 3-phosphoenol pyruvate LOD was one of the lowest at 0.09 μM with a 20 μL injection volume. Somewhat higher LODs were observed for some intermediates, such as ribulose 5-phosphate and dihydroxyaceton 1-phosphate. The calibration curve for each compound was computed by plotting the peak intensity ratios between variable (light) and constant (heavy) amount of standard versus the additive nominal concentrations. Linearity was calculated using a nonweighted least-squared linear regression method and generally spanned 2−3 orders of magnitude with correlation coefficients higher than 0.995. A linear regression of all compounds showed a unit slope with interception close to zero (data not shown). Analytical precision was calculated from within-assay variability by measuring the peak intensity ratio of the analyte to its $^{13}C_6$-reference at a concentration ratio of 1:1, and expressed as the percentage relative standard deviation. The variation was generally

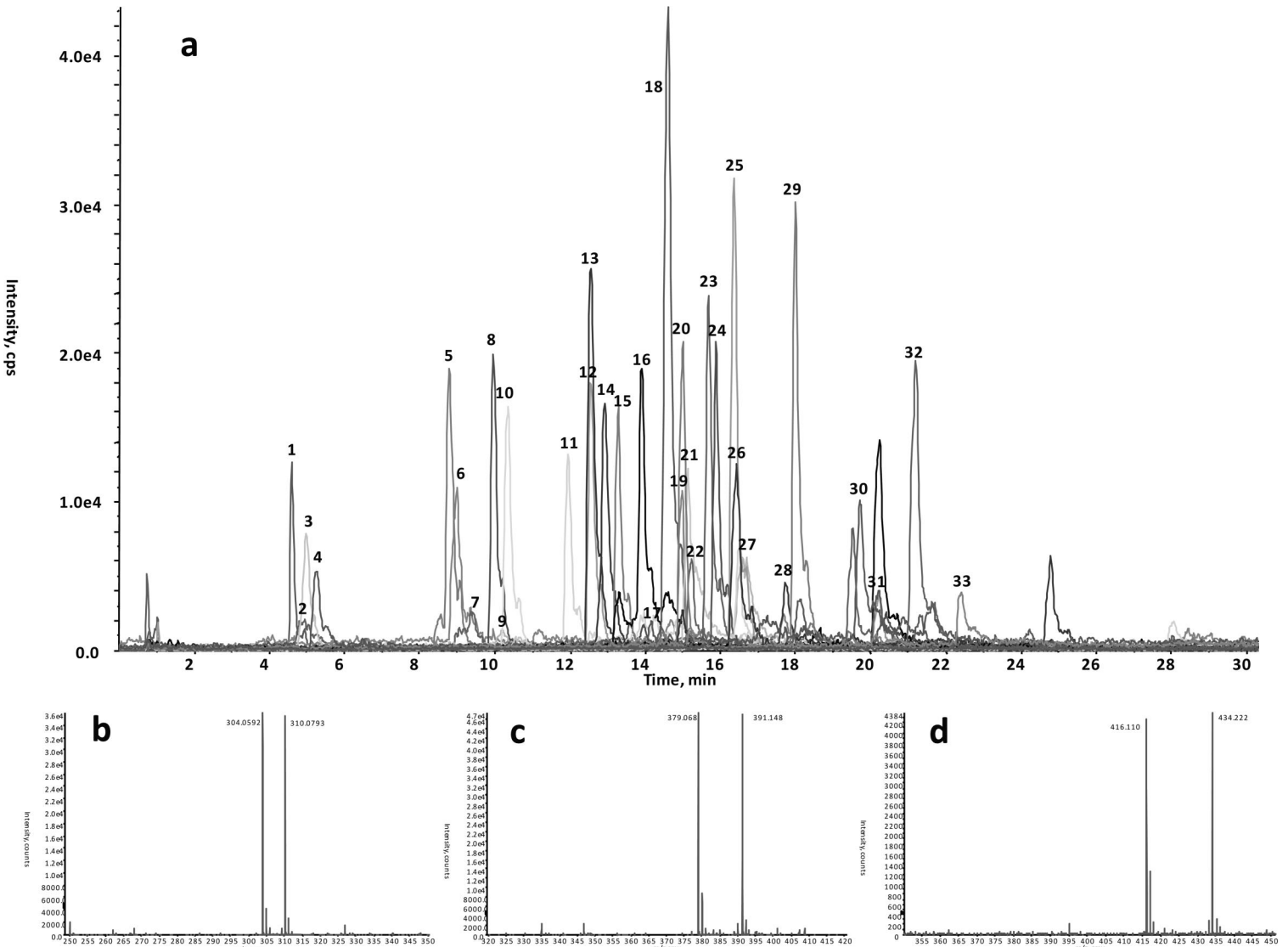

Figure 3. Overlapped extracted ion chromatograms of 33 metabolites in a 14.3 μM standard mixture. Peak identification is given in Table 1. LC−MS conditions are described in the Experimental Section (a). Representative MS doublets from aniline-labeled and aniline-$^{13}C_6$-labeled metabolites which are mixed at 1:1 ratio: D-ribulose 5-phosphate-monoaniline (b); D-ribose 5-phosphate-bisaniline (c); citrate trianiline (d).

Table 2. Basic Parameters of the Method

peak no.	compd	LOD (μM)[a]	LOQ (μM)[b]	$^{12}C/^{13}C$ linearity range[c]	R^2	RSD (%, $n = 5$)[d]
1	glycerol 3-phosphate[e]	0.48	1.68	N/A	N/A	N/A
2	xylose	2.51	7.88	1:1 to ~1/240:1 (1.00)	0.9968	5.08
3	NAD[e]	0.77	2.40	N/A	N/A	N/A
4	glucose	1.15	3.97	1:1 to ~1/240:1 (0.80)	0.9972	4.79
5	fructose 6-phosphate	0.31	1.03	1:1 to ~1/800:1 (0.26)	0.9955	1.21
6	lactic acid	0.57	1.93	1:1 to ~1/800:1 (0.80)	0.9984	1.10
7	D-ribulose 5-phosphate	2.51	8.51	1:1 to ~1/400:1 (1.00)	0.9977	3.23
8	AMP	0.30	1.04	1:1 to ~1/800:1 (0.26)	0.9958	0.89
9	dihydroxyacetone 1-P	2.50	8.23	1:1 to ~1/400:1 (1.00)	0.9965	4.12
11	D-(−)glycerate 3-phosphate	0.47	1.45	1:1 to ~1/800:1 (0.40)	0.9988	1.35
12	FAD[e]	0.31	1.04	N/A	N/A	N/A
13	ADP	0.28	0.98	1:1 to ~1/800:1 (0.26)	0.9980	1.02
14	fructose 1,6-bisphosphate	0.35	1.17	1:1 to ~1/600:1 (0.26)	0.9966	2.38
15	gluconate 6-phosphate	0.35	1.22	1:1 to ~1/800:1 (0.26)	0.9954	2.17
16	glucose 6-phosphate	0.41	1.37	1:1 to ~1/800:1 (0.26)	0.9969	2.93
17	NADH	2.51	9.03	1:1 to ~1/400:1 (1.00)	0.9972	4.29
18	ketoglutarate	0.12	0.42	1:1 to ~1/800:1 (0.26)	0.9994	0.98
19	DL-glyceraldehyde 3-P	0.55	1.80	1:1 to ~1/600:1 (0.40)	0.9981	3.97
20	malate	0.30	1.06	1:1 to ~1/800:1 (0.26)	0.9975	2.15
21	ATP	0.55	1.77	1:1 to ~1/600:1 (0.40)	0.9983	1.17
22	D-ribose 5-phosphate	0.62	2.05	1:1 to ~1/800:1 (0.80)	0.9979	3.01
23	acetyl Co A[e]	0.24	0.86	N/A	N/A	N/A
24	D-erythrose 4-phosphate	0.31	1.07	1:1 to ~1/800:1 (0.26)	0.9962	3.28
25	phospho(enol)pyruvate	0.09	0.32	1:1 to ~1/800:1 (0.26)	0.9974	1.36
26	succinate	0.46	1.55	1:1 to ~1/800:1 (0.40)	0.9955	3.45
27	NADPH	0.61	1.88	1:1 to ~1/600:1 (0.40)	0.9960	3.52
28	fumarate	1.22	4.17	1:1 to ~1/160:1 (0.80)	0.9982	3.42
29	glycerate 1,3-bisphosphate	0.19	0.66	1:1 to ~1/800:1 (0.26)	0.9963	1.71
30	oxalacetate	0.55	1.75	1:1 to ~1/320:1 (0.40)	0.9978	4.42
31	isocitrate	1.30	4.47	1:1 to ~1/320:1 (0.80)	0.9957	3.99
32	citrate	0.31	1.11	1:1 to ~1/320:1 (0.40)	0.9964	4.53
33	*cis*-aconitate	1.32	4.77	1:1 to ~1/320:1 (0.80)	0.9976	4.65

[a] Based on peak intensity at S/N = 3. [b] Based on peak intensity at S/N = 10. [c] Concentration ratio between $^{12}C_6$-aniline-labeled and $^{13}C_6$-aniline-labeled analyte at the constant concentration of $^{13}C_6$-aniline-labeled analyte (indicated in the parentheses). [d] RSD is for the peak intensity ratio of the $^{12}C_6$-aniline-labeled to the $^{13}C_6$-aniline-labeled at the concentration ratio of 1:1. [e] Not labeled compounds.

below 5%. This approach reflects variations from the whole process, including sample preparation, labeling, and LC−MS analysis.

Determination of Metabolites in Yeast Cell Extract. In this study we have focused on 35 intermediates involved in yeast central carbon and energy metabolism. Due to unavailability of standards or structural analogues five metabolites could not be quantified. The rest of metabolites can be divided into the three categories based on the quantification approach.

Labeled Metabolites (Approach A). Typically, GSIST quantification includes derivatization of sample and standard solutions with aniline and aniline-$^{13}C_6$, respectively. After labeling, mixtures of the sample and standards are mixed at a specific ratio and analyzed by LC−MS. The concentration of individual metabolites is then determined from the ratio between the intensity of corresponding light (sample) and heavy (standard) peaks in the doublet sets of ions. A potential problem with this approach is that when the background matrix in the sample and standard mixture is very different derivatization efficiency might be impacted. A further refinement of the methods described above that would deal with this issue is a labeling and quantification schema referred to as global isotope-labeled internal standard addition (GILISA).

The principle of GILISA is described in the equation

$$V_x C_x/(V_x C_x + V_a C_a) = F_r I_x/I_{x+a} \quad (1)$$

where C_x and C_a are the concentration of the analyte and added standard, V_x and V_a are the volumes corresponding to the analyte and standard, I_x and I_{x+a} are the signal intensity of an unknown analyte and the signal intensity after addition, and F_r is an instrument response factor. Typically the instrument response factor, F_r, is assumed to be 1,[16,19,31] i.e., there is no variation in instrument response between metabolites and standards. The exception to this would be when there is variable matrix suppression of ionization.

In GSIST, factor F_r represents both instrument variation as well as the difference in derivatization efficiency and can be calculated when standards are added into two samples in equal concentration according to the equation. This is represented by the equation

$$F_r = R_{x+a}/R_x \quad (2)$$

where R_x and R_{x+a} represents signal intensity of the ^{13}C-labeled standards in the ^{12}C-labeled unknown sample and in the ^{12}C-labeled unknown sample spiked with known amount of the standards, respectively. Combining eqs 1 and 2 produces the formula for GILISA where C_x is the concentration of the unknown.

$$C_x = R_{x+a} I_x/(R_x I_{x+a} - R_{x+a} I_x)(V_a/V_x) C_a \quad (3)$$

(31) Sekiguchi, Y.; Mitsuhashi, N.; Kokaji, T.; Miyakoda, H.; Mimura, T. *J. Chromatogr., A* **2005**, *1085*, 131–136.

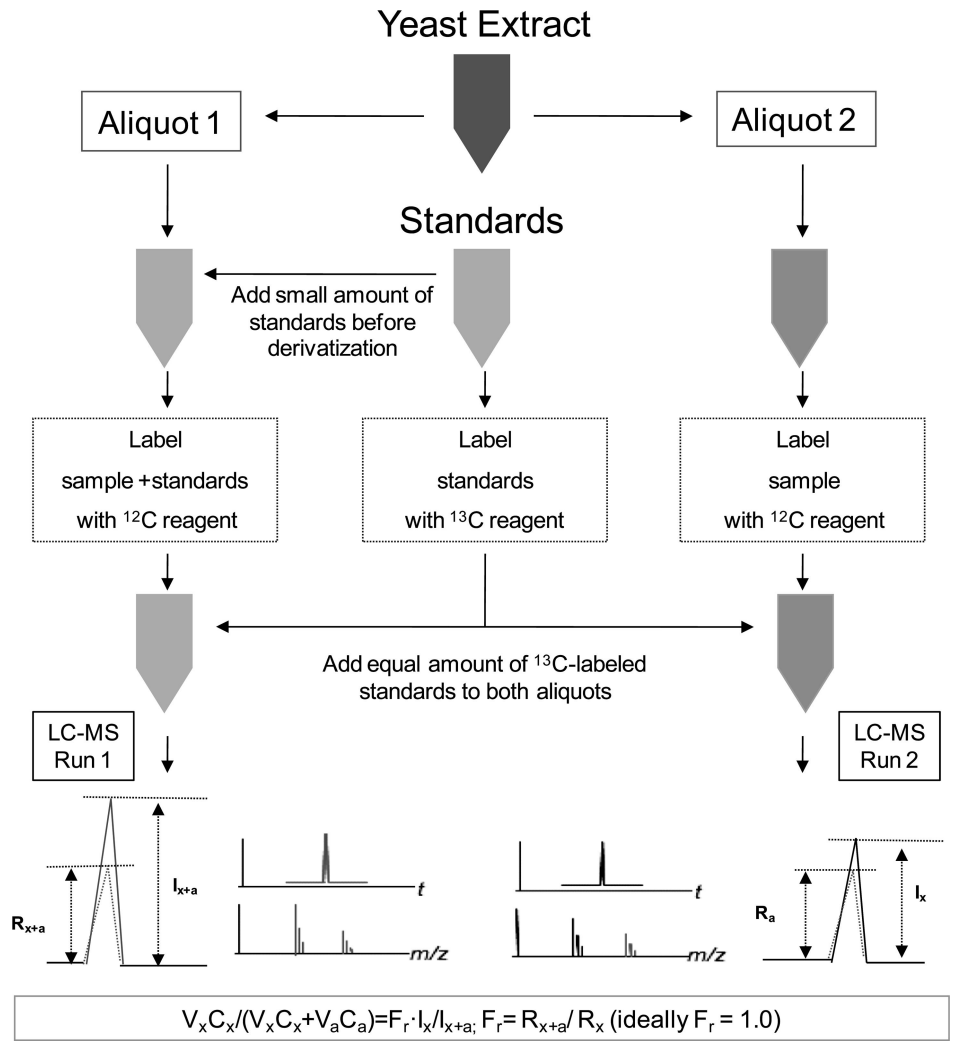

Figure 4. Workflow for metabolite identification and quantification by GILISA.

The workflow for the method is described in Figure 4. Two 100 μL aliquots of yeast extract were taken to which 10 μL of 286 μmol/L standard mixture was added to one of them. These two aliquots were labeled with aniline. In the meantime, a 100 μL aliquot of the standard mixture containing 14.3 μmol/L of each analyte was labeled with aniline-$^{13}C_6$. After labeling, 50 μL of this aniline-$^{13}C_6$-labeled standard mixture was added into the above two aniline-labeled samples. After LC−MS analysis, the quantification was achieved according to eq 3.

Most of the intermediates in the cell extract were quantified in this manner, but as noted some of the metabolites were not labeled or standards were not available. In the *labeled but no standard available approach (B)*, quantification of D-6-phosphoglucono-δ-lactone was based on the ^{13}C-labeled standard of its contiguous peak, fructose 6-phosphate, which served as structural analogue. With the *unlabeled metabolites approach (C)*, underivatized analytes were quantified by standard addition as described by Huck et al.[19]

The quantification results of yeast central carbon and energy metabolism are summarized in Figure 5. Generally, the RSD was below 10.0%.

CONCLUSIONS

Prior to the advent of metabolomics, classes of metabolites were generally examined individually as was the case with adenosine phosphate,[32] sugar phosphates,[11,19,22,31] or carboxylic acids from the TCA cycle.[33−37] Though some methods worked on multiple classes,[12,13,38] the coverage was not complete.

The intent in this work was to analyze multiple classes of intermediates, including nucleotides and cofactors directly involved in central carbon metabolism by RPLC−MS in a single run. Among the numerous problems were that some unlabeled analytes showed little or no retention in RPLC. Another problem was that metabolite pools were highly complicated and some analytes were present in low abundance. Another complication was that some classes of analytes were structurally very similar and could not be differentiated by MS. Among the problem metabolites were glucose 6-phosphate and fructose 6-phosphate,

(32) Enrich, M. J. D.; Barcia, R.; Martinez, J. I. R.; Ibarguren, I. *J. Liq. Chromatogr. Relat. Technol.* **1999**, *22*, 1391–1402.
(33) Kubota, K.; Fukushima, T.; Yuji, R.; Miyano, H.; Hirayama, K.; Santa, T.; Imai, K. *Biomed. Chromatogr.* **2005**, *19*, 788–795.
(34) Masson, S.; Sciaky, M.; Desmoulin, F.; Fontanarava, E.; Cozzone, P. J. *J. Chromatogr.* **1991**, *563*, 231–242.
(35) Tatar, E.; Mihucz, V. G.; Varga, A.; Zaray, G.; Fodor, F. *Microchem. J.* **1998**, *58*, 306–314.
(36) Womersley, C.; Drinkwater, L.; Crowe, J. H. *J. Chromatogr.* **1985**, *318*, 112–116.
(37) Zielinska, D.; Poels, I.; Pietraszkiewicz, M.; Radecki, J.; Geise, H. J.; Nagels, L. J. *J. Chromatogr., A* **2001**, *915*, 25–33.
(38) Bajad, S. U.; Lu, W.; Kimball, E. H.; Yuan, J.; Peterson, C.; Rabinowitz, J. D. *J. Chromatogr., A* **2006**, *1125*, 76–88.

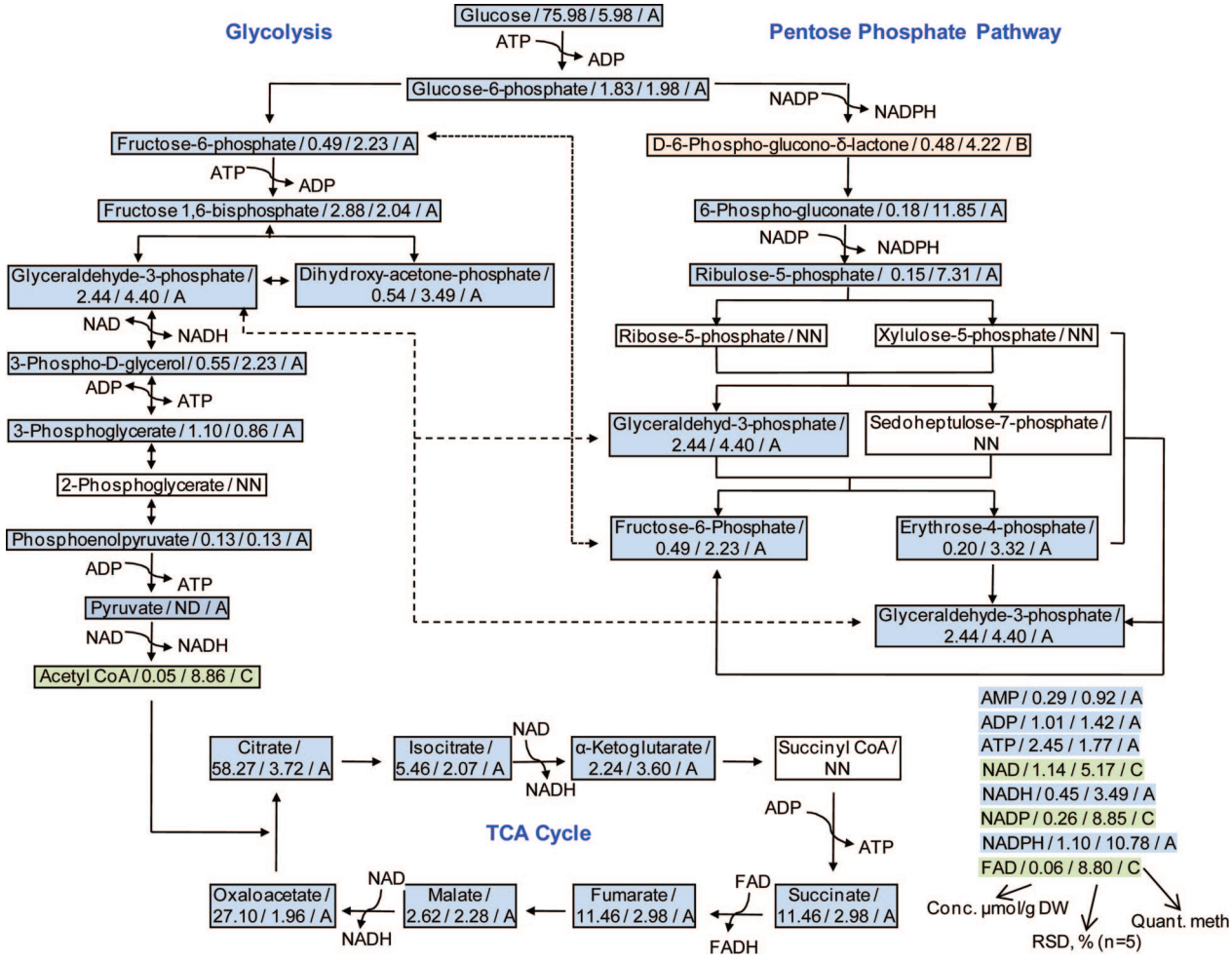

Figure 5. Central carbon metabolism map and the determined metabolite concentration in yeast. Quantification approach using A—GILISA, B—structural analogue, and C—standard addition. NN, no standard available; ND, not determined.

ribose 5-phosphate and ribulose 5-phosphate, glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, citrate and isocitrate. The only way to differentiate between them was by chromatographic resolution, when possible. Still another issue was that ^{13}C-labeled standards are not available for accurate MS-based quantification in some cases.

A robust RPLC−MS method is described here that allows accurate determination of essentially all the intermediates involved in central carbon metabolism in a single 30 min run through in vitro labeling with isotopically distinguishable coding agents. (Figure 5). The only exception was pyruvate. In vitro isotopic coding with aniline provides several benefits. By introducing a hydrophobic moiety into hydrophilic molecules, hydrophobicity is increased, facilitating both reversed-phase separation and ESI-MS detection. Moreover, the different labeling patterns for aldose sugars and ketose sugars simplify their separation. In vitro isotopic coding provides additional criterion for the identification of metabolites in complex matrixes by producing an easily recognizable doublet ion pattern. Even though in vivo isotope coding can also be used,[39] it is not possible to control the chemical nature of the appended labeling agent. Being able to add hydrophobicity during isotope coding was a great analytical asset. The labeling also offers an opportunity to accurately quantify metabolites by MS, which is usually difficult if their $^{13}C_6$-coded standards are not available. This approach opens a door for comparative quantification of multiple analytes in a single run. In some studies, it is likely that one would compare the concentration of metabolites in a sample under one set of biological conditions with concentration found under another set of conditions. It can be simply done by labeling two samples with $^{13}C_6$-coded and non-$^{13}C_6$-coded aniline, respectively, and then two labeled samples are mixed equally. By MS, two labeled samples can be visually and quantitatively compared.

(39) Lu, W.; Kimball, E.; Rabinowitz, J. D. *J. Am. Soc. Mass Spectrom.* **2006**, *17*, 37–50.

ACKNOWLEDGMENT

This work was supported by Grants from the U.S. Department of Energy Biomass Program (GO17059-16649) and the National Science Foundation (DBI-0421102). We also thank C. Huetteman for discussions and critical reading of the manuscript.

Received for review August 12, 2008. Accepted October 22, 2008.

AC801693C