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Streamlined pentafluorophenylpropyl column liquid chromatography–tandem quadrupole mass spectrometry and global ^{13}C-labeled internal standards improve performance for quantitative metabolomics in bacteria

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ABSTRACT

Streamlined quantitative metabolomics in central metabolism of bacteria would be greatly facilitated by a high-efficiency liquid chromatography (LC) method in conjunction with accurate quantitation. To achieve this goal, a methodology for LC–tandem quadrupole mass spectrometry (LC–MS/MS) involving a pentafluorophenylpropyl (PFPP) column and culture-derived global ^{13}C-labeled internal standards (I.Ss.) has been developed and compared to hydrophilic interaction liquid chromatography (HILIC)–MS/MS and published combined two-dimensional gas chromatography and LC methods. All 50 tested metabolite standards from 5 classes (amino acids, carboxylic acids, nucleotides, acyl-CoAs and sugar phosphates) displayed good chromatographic separation and sensitivity on the PFPP column. In addition, many important critical pairs such as isomers/isobars (e.g. isoleucine/leucine, methylsuccinic acid/ethylmalonic acid and malonyl-CoA/3-hydroxybutyryl-CoA) and metabolites of similar structure (e.g. malate/fumarate) were resolved better on the PFPP than on the HILIC column. Compared to only one ^{13}C-labeled I.S., the addition of global ^{13}C-labeled I.Ss. improved quantitative linearity and accuracy. PFPP-MS/MS with global ^{13}C-labeled I.Ss. allowed the absolute quantitation of 42 metabolite pool sizes in *Methylobacterium extorquens* AM1. A comparison of metabolite level changes published previously for ethylamine (C2) versus succinate (C4) cultures of *M. extorquens* AM1 indicated a good consistency with the data obtained by PFPP-MS/MS, suggesting this single approach has the capability of providing comprehensive metabolite profiling similar to the combination of methods. The more accurate quantification obtained by this method forms a fundamental basis for flux measurements and can be used for metabolism modeling in bacteria in future studies.

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1. Introduction

Metabolomics is growing in importance as one field of systems biology, as it has great potential for providing insights into the activity of cells in response to genetic or environmental perturbation. Almost all metabolomics research falls into one of two categories: metabolite profiling and quantitative metabolomics. In combination with chemometrics tools, metabolic profiling has been successful in describing cell phenotypes and identifying biomarkers between different strains, tissues or growth conditions [1–3]. By contrast, quantitative metabolomics can directly determine dynamic metabolite pool sizes and thus plays an increasingly important role in elucidating *in vivo* reaction kinetics, in metabolic modeling, and in predicting metabolic flux and modeling [4–6].

Mass spectrometry (MS)-based metabolomics, in which a separation technique such as gas chromatography (GC), capillary electrophoresis (CE) or liquid chromatography (LC) is coupled to a mass spectrometer, has been widely applied to profile metabolomes or determine metabolite concentrations [7–9]. Due to the versatile separation characteristics of LC, broader selectivity, and omission of derivatization steps, LC–MS is often the preferred technique for metabolomic analysis. Metabolites are typically moderately to highly polar small molecules, which are often too hydrophilic to be reliably retained and separated on common reversed-phase columns (RPLC). Ion-pairing reverse phase chromatography is gaining popularity for metabolomics applications, as it enables retention and resolution of polar metabolites on the reverse phase [10–12], but the high background of the mobile phase can be an issue in a facility without ded-

Abbreviations: PFPP-MS/MS, LC–MS/MS using PFPP column.

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icated instrumentation for ion paring chromatography [10,12]. The hydrophilic interaction liquid chromatography (HILIC) technique provides an advance in retention and separation of polar compounds, often with improved sensitivity due to the high percentage of the organic mobile phase. Generally HILIC analysis is suitable for various compound classes including nucleotides, carboxylic acids, amino acids and small peptides etc. [13]. However, some metabolites in these classes have similar physicochemical properties, such that full separation and reliable quantification of compounds such as malate/fumarate, leucine/isoleucine and succinyl-CoA/methylmalonyl-CoA were usually challenging for typical HILIC analyses [14,15]. As a result, the combination of multiple LC-based and GC-based methods for the same sample was preferred to increase the resolution of metabolites [16,17]. In a recent report, we have investigated the central metabolism of *Methylobacterium extorquens* AM1 by using a combination of complementary separation techniques (RPLC, HILIC and comprehensive two-dimensional gas chromatography (GC × GC)) with MS [18]. It was found that although a high number of metabolites were identified in the cells, data processing for quantification, especially the automatic analysis of GC × GC–time-of-flight (TOF)-MS data, was complicated when the ^{13}C-labeled internal standards (I.Ss.) were introduced prior to cell extraction. To obtain both better coverage and reliable quantification of the metabolome, another chromatographic approach was needed.

Fluorinated silica-based stationary phases have been introduced and applied to analyze small polar analytes over the last 10 years [19,20]. In particular, pentafluorophenylpropyl (PFPP) phases attaching a pentafluorophenyl ring structure to the silica over a propyl chain exhibited both reversed- and normal-phase retention and were shown to offer a different selectivity for polar compounds compared to traditional alkyl phases and HILIC phases [21]. The retention mechanism has been described as a reversed-phase mode with an alkyl chain, and an ion-exchange-mode with an ionized surface silanol as well hydrogen bonding and π–π interactions [21–23], but relatively few applications of the PFPP phase for quantitative metabolomics have been reported [22].

In addition to a good retention and separation of metabolites, introduction of I.S. to the samples prior to metabolite extraction is important for reliable quantification. When complex biological extracts are injected into an electrospray ionization (ESI) source, the ionization efficiency of metabolites can be suppressed or enhanced due to the presence of less volatile and coeluting compounds [24]. By adding the ^{13}C-labeled I.Ss., especially cell culture derived global ^{13}C-labeled I.Ss. to the samples, corrections can be made for the variations arising from instrumental analysis and sample preparation [25–28].

For the current work we have applied the new method to study the metabolome of the facultatively methylotrophic bacterium *M. extorquens* AM1, which is already well studied as a model organism for understanding one-carbon metabolism under the context of the genome and proteome [29,30]. The changes of central metabolites of *M. extorquens* AM1 grown on different carbon sources (C2 substrate, ethylamine; C4 substrate, succinate) were analyzed and compared to the previously published combined method [18]. Although the combined method provided a possibility for simultaneous analysis of various classes of metabolites, it was at the expense of increasing experimental operation and complicating data processing. Our goal was to develop a suitable high-efficiency liquid chromatography and accurate quantitation method for analysis of central metabolites in a biological sample. In order to do so, a streamlined method utilizing PFPP column LC–MS/MS has been applied to determine diverse classes of metabolites in a single method. This new improved analytical approach was combined with a culture-derived global ^{13}C-labeled I.Ss. for reliable absolute quantification in *M. extorquens* AM1.

2. Experimental

2.1. Chemicals

The standards of nucleotides, acyl-CoAs, amino acids, organic acids and sugar phosphates were of analytical grade and obtained from Sigma (St. Louis, MO, USA). Absolute ethanol (EMD Chemicals, Gibbstown, NJ, USA) was used in the cell extraction. Solvents used for liquid chromatography were of LC–MS grade (Fisher Scientific, Fair Lawn, NJ, USA). Millipore purified water was used in the preparation of standards and sample solutions. ^{13}C-MeOH (99%) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). For GC–MS sample derivatization, pyridine was purchased from EMD Chemicals, and the trimethylsilylation (TMS) reagent (*N*,*O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) + Trimethylchlorosilane (TMCS), 99:1) and methoxyamine hydrochloride were both obtained from Sigma.

2.2. Batch growth conditions of *M. extorquens* AM1 and continuous cultivation of ^{13}C-labeled biomass

M. extorquens AM1 (rifamycin-resistant strain) was grown in liquid batch cultures in a minimal medium as described previously [31]. Ethylamine (20 mM) and succinate (15 mM) were used as the substrates. Rifamycin was added at 50 μg/mL and the culture temperature was maintained at 28 °C.

Continuous cultivation of *M. extorquens* AM1 was carried out using a 1-L bench-top BioFlo 110 Modulator Fermentor (New Brunswick Scientific, Edison, NJ, USA) with a 600 mL working volume [32]. The same mineral medium was used as in batch cultivation, with 25 mM ^{13}C-MeOH as the sole carbon source. The CO_2 in the air supply was removed during cultivation by passing the air through three 1-L bottles containing 4 M KOH solution connected in series. The dilution rate was maintained at 0.1 h^{-1} and the optical density (OD_{600}) at steady-state was 1.0 ± 0.1 for harvesting ^{13}C-labeled biomass.

2.3. Preparation of samples

2.3.1. Preparation of global ^{13}C-labeled cell extracts

10 mL of each ^{13}C-labeled cell culture was carefully and rapidly pipetted into the center of 28 mL 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered (70 mM, pH 6.8) aqueous 60% methanol (v/v) solution (−40 °C). The quenched biomass was spun down in a refrigerated centrifuge (10 min, 10,000 rpm, −20 °C; Dupont Sorvall RC5B, Waltham, MA, USA). The supernatant was removed, and the cell pellets were resuspended in 5 mL of the same cold methanol solution and again centrifuged for 6 min at 10,000 rpm. Cell pellets were used for the extraction of global ^{13}C-labeled I.Ss. as described below.

The nucleotides, carboxylic acids, amino acids and sugar phosphates were extracted by using a previously reported protocol [18]. Briefly, 1.5 mL of boiling HEPES buffered ethanol solution (75% (v/v) ethanol/water, pH 5.2) was added to a given cell pellet and incubated at 100 °C for 5 min. The cell debris was removed by centrifugation at 5000 rpm for 5 min. The cell-free metabolite extract was again centrifuged at 14,000 rpm for 8 min. The supernatant was dried in a vacuum centrifuge (CentriVap Concentrator System, Labconco, MO, USA) to complete dryness and stored at −80 °C. For the LC–MS/MS analysis the dried sample was redissolved in 100 μL purified water and pooled with acyl-CoAs cell extracts described below. The dried sample that was analyzed by GC–MS was further derivatized in two steps as described previously [18] except the methoximation step was carried out at 30 °C for 90 min.

For the short-chain acyl-CoAs, an acid extraction method previously described [18] was used. Briefly, 0.8 mL trichloroacetic acid

(TCA) (15%, w/v) was added to a given cell pellet and vortexed 5 min at 4 °C. The mixture was then centrifuged at 14,000 rpm for 5 min. The supernatant was loaded onto a solid-phase extraction cartridge (Waters Oasis HLB 1 cc). The elution solvent was evaporated to dryness using a vacuum centrifuge. The dried samples were mixed with 100 μL of sample solution obtained from the hot ethanol extraction.

All ^{13}C-labeled cell extracts were then pooled, diluted 2 times, aliquoted and stored at −80 °C for further use.

2.3.2. Preparation of cell culture samples

6 mL of cell culture at OD_{600} = 1.0 ± 0.2 was quenched in 20 mL of cold methanol solution (HEPES-buffered (70 mM, pH 6.8) aqueous 60% methanol (v/v) solution (−40 °C)) as described above. After quenching, a fixed amount (90 μL) of global ^{13}C-labeled I.Ss. was rapidly added to cell pellets prior to the cell extraction. The metabolite classes were extracted separately as described above. The dried sample was dissolved in 100 μL purified water and diluted 2 times with 0.2% formic acid water for the LC–MS/MS analysis.

2.4. LC–MS/MS analysis

LC–MS/MS experiments were carried out on a Waters (Milford, MA, USA) LC–MS system consisting of a 1525μ binary HPLC pump with a 2777C autosampler coupled to a Quattro Micro API triple-quadrupole mass spectrometer (Micromass, Manchester, UK).

LC solvents for the HILIC method were carried out using the previously described column (Luna NH2, 250 mm × 2 mm, 5 μm, Phenomenex, Torrance, CA, USA) and conditions with minor modification [14]. The mobile phase A consisted of 20 mM ammonium acetate + 0.05% (v/v) ammonium hydroxide (28%) in water/acetonitrile (95:5, v/v), pH 8.50, while mobile phase B was acetonitrile. The following linear gradient was used: 85–0% B for 15 min, 0% B for 4 min, 0–85% B for 1 min, 85% B for 15 min. The total run time was 35 min at 0.15 mL/min. For the separation on the PFPP column (Luna PFP (2), 150 mm × 2 mm, 3 μm, Phenomenex, Torrance, CA, USA) the mobile phase A was water with 0.1% (v/v) formic acid and mobile phase B was acetonitrile. For the analytes detected in positive ESI mode, a linear gradient was used: 0% B for 8 min, 0–25% B for 18 min, 25–100% B for 2 min, 100% B for 5 min, 100–0% B for 1 min and 0% B for 6 min. For the analytes detected in negative ESI mode, a linear gradient was used 0% B for 8 min, 0–30% B for 7 min, 30–50% B for 1 min, 50% B for 5 min, 50–100% B for 1 min and 100% B for 5 min, 100–0% B for 1 min and 0% B for 6 min. The total run time was 40 min and 34 min at 0.20 mL/min, respectively. The injection volume was 10 μL for both methods. The system hold-up time of the PFPP column was estimated by monitoring the first UV peak (UV 220 nm) upon an acetone injection. The hold-up time was approximately 1.4 min. The eluent from the LC column was directed into the ion source of mass spectrometer.

The MS/MS experiments were carried out as described previously [18]. The multiple reaction monitoring (MRM) precursor/product ion pairs for ^{12}C and ^{13}C metabolites are listed in Supplemental Table 1. The dwell time for each MRM transition was 0.1 s. All peaks were integrated using the Masslynx Quanlynx Applications Manager (version 4.1) software.

2.5. GC/MS analysis of ^{13}C incorporation

GC/MS experiments were performed using an Agilent 5973 MSD/6890 GC (Agilent Corp, Santa Clara, CA, USA). The column was Rtx-5MS (30 m × 0.25 mm × 0.5 μm film, Restek, Bellefonte, PA, USA). Ultra high purity helium was used as a carrier gas at a constant flow mode of 1 mL/min, and 1 μL of a given sample was injected in split-less mode via an Agilent 7683 autosampler. The inlet temperature was set at 280 °C. The temperature program began at 60 °C with a hold time of 0.25 min, then increased at 8 °C/min to 280 °C with a hold time of 10 min at 280 °C. The ion source temperature was set to 250 °C. Mass spectra were collected from m/z 40 to 400 at 3 spectra/s with a 6 min solvent delay. The peaks were analyzed using Agilent data analysis software.

2.6. Preparation of calibration curve and statistical analysis

The calibration curve of standard mixtures was obtained by analyzing standard solutions at eight concentrations ranging from 200 nM to 50 μM (200, 500, 1000, 2500, 5000, 10,000, 25,000, 50,000 nM). For glutamine and glutamic acid the calibration range was extended to 400 μM.

The calibration curve with addition of ^{13}C-labeled I.Ss. was prepared by mixing different dilutions of primary stock solutions of standard mixtures with fixed amounts of cell-derived global ^{13}C-labeled I.Ss. For each dilution, 4 μL of standard mixtures was added to 36 μL ^{13}C-labeled cell extracts, making the final concentration of standards as described above. The calibration curve and correlation coefficient (R^2) were obtained by a linear regression from the plot of the ratio of standard peak area (A_{un}) to the area of fully ^{13}C-labeled internal standard (A_{ful}) versus concentration of standards.

Independent two-sample t-tests were performed on the metabolomic datasets from the growth conditions succinate and ethylamine using Microcal Origin, 5.0. The false discovery rate (FDR) procedure of Benjamini and Hochberg [33] as implemented in R (R development Team, 2008) was used to correct for the multiple comparisons problem. These adjusted FDR were used to assign significance at a FDR of 0.05.

3. Results

3.1. PFPP phase improved the separation of primary metabolites

In the initial experiments, 50 primary metabolites from 5 classes (amino acids, carboxylic acids, nucleotides, acyl-CoAs and sugar phosphates) commonly involved in central metabolism of bacteria were targeted for investigating the performance of HILIC chromatography. These 5 classes of metabolites exhibited great diversity with respect to molecular weight, polarity and pKa value, challenging the chromatographic separation. All of the metabolites were retained significantly beyond the void time under the current HILIC conditions (Supplemental Table 1). The metabolites which are isobars/isomers or have similar fragment ions and close retention were considered to be critical pairs for the separation. The following critical pairs were identified: lysine/glutamine; glutamine/glutamic acid; leucine/isoleucine; methylsuccinic acid/ethylmalonic acid; succinic acid/methylmalonic acid; malate/fumarate; citric acid/isocitric acid; butyryl-CoA/acetyl-CoA; succinyl-CoA/methylmalony-CoA; 3-hydroxybutyryl-CoA/malonyl-CoA; fructose-6-phosphate/glucose-6-phosphate (F6P/G6P). On the HILIC column, it was observed that lysine/glutamine, glutamine/glutamic acid and methylsuccinic acid/ethylmalonic acid were separated but the other critical pairs such as leucine/isoleucine, succinic acid/methylmalonic acid and citric acid/isocitric acid were not clearly distinguished (Fig. 1). For malate and fumarate, having similar chemical structure and very close retention times (Fig. 1B), an in-source loss of water from malate could contribute to a fumarate MS signal intensity. In addition, we found it difficult to resolve all of the acyl-CoA compounds and peak tailing was observed for some of acyl-CoAs (data not shown).

To resolve critical pairs and obtain resolution for the other metabolites, a PFPP (pentafluorophenylpropyl) column was implemented. While the PFPP column can perform in the normal phase mode, 85% acetonitrile (mobile phase B) as an initial condition did

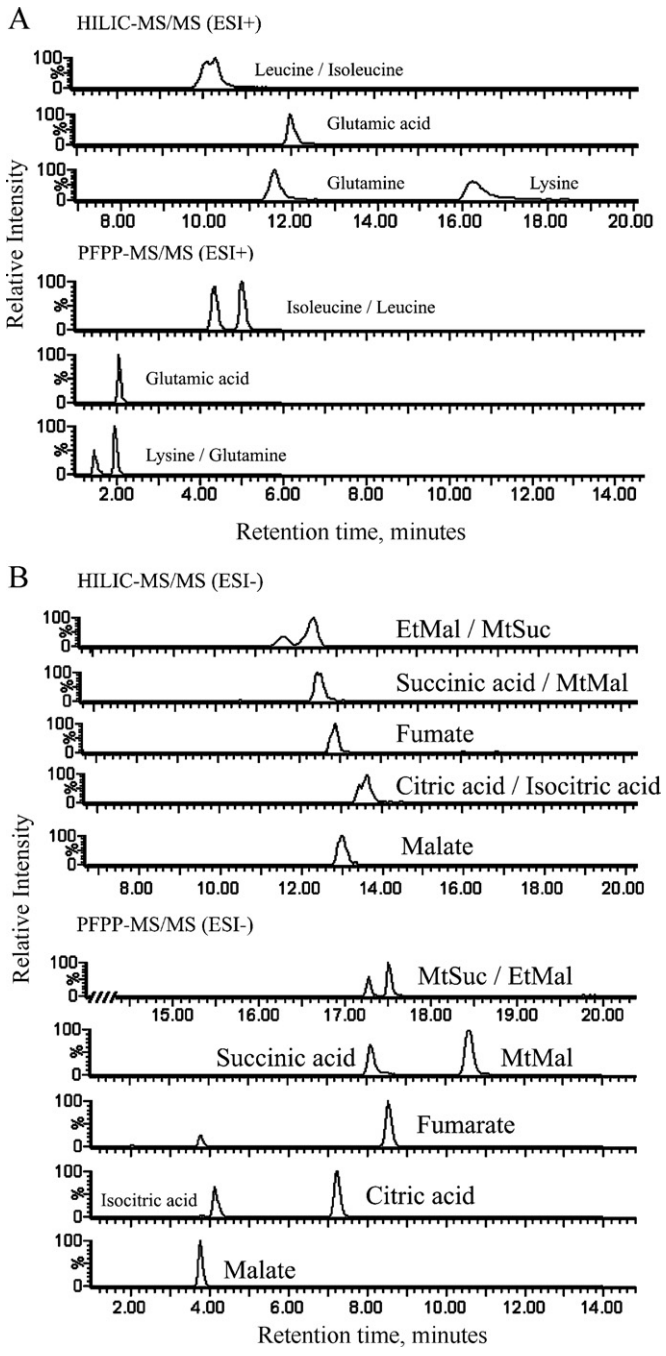

Fig. 1. The typical HILIC and PFPP chromatograms for the separation of critical pairs of amino acids (A) and carboxylic acids (B). The standard mixtures were 5 μM of each compound. The abbreviations used are EtMal, ethylmalonic acid; MtSuc, methylsuccinic acid; MtMal, methylmalonic acid.

not retain and separate all analytes sufficiently. In contrast, reverse phase mode gradient elution on the PFPP column separated the metabolites. The buffer additive in water affected the separation on the PFPP column most. When used with 20 mM ammonium formate (pH 3.0) or ammonium acetate buffer (pH 4.5), significantly lower overall retention and poor isomer separation was observed (data not shown). The best results were obtained with 0.1% aqueous formic acid/acetonitrile gradient which provided good retention (retention factor, $1 < k' < 20$) for most tested metabolites. Most of the 5 classes of metabolites were observed with good peak shape, a typically symmetrical peak around 30 s wide except for broader peaks for pyruvate and α-ketoglutaric acid, and ATP peak tailing. All 5 classes of metabolites were reproducibly separated in a time window from 2 to 30 min (Fig. 1 and Supplemental Table 1). Good separation can reduce the potential effects of ion suppression from co-eluting components. The variation of retention time ranged from 0.5% to 3.2% on the PFPP based on multiple injections, but the variation on the HILIC was up to 5.1% (Supplemental Table 1). One possible explanation is that HILIC separation is more sensitive to the buffer pH which can vary due to the mobile phase having been prepared fresh every day.

As shown in Fig. 1, during the first 8 min of isocratic elution, isomers/isobars such as lysine/glutamine, leucine/isoleucine, citric acid/isocitric acid, and succinic acid/methylmalonic acid were fully resolved ($R > 1.5$). Notably, malate and fumarate were separated by more than 3 min to increase their accuracy of quantification. After the initial isocratic portion, the isomers of methylsuccinic acid and ethylmalonic acid were successfully separated under gradient elution (Fig. 1B). The shallow gradient separated all the acyl-CoAs including isomer/isobar (Fig. 2A). The peaks for malonyl-CoA/3-hydroxybutyryl-CoA and succinyl-CoA/methylmalonyl-CoA having the same MRM transition due to the loss of the CoA-specific fragment ion ($m/z = 428$) were baseline resolved. Fig. 2B shows seven important nucleotides well separated under the optimized chromatographic condition. Fructose-6-phosphate and glucose-6-phosphate were partially resolved under the reported conditions (Supplementary Table 1).

Next the sensitivity of the PFPP-MS/MS and HILIC–MS/MS methods were evaluated. A calibration curve for pure standard mixtures was obtained by analyzing standard solutions at eight concentrations ranging from 200 nM to 50 μM. The limit of detection (LOD) (defined as the lowest concentration at which the signal-to-noise ratio is larger than 3) was determined based on a calibration curve for each metabolite (Table 1). The observed LODs for all the amino acids were lower than 1 pmol on column, and in some cases the sensitivity improved 3–15 times compared to HILIC–MS/MS. The LODs of the acyl-CoAs were below 1.5 pmol on the PFPP phase. The LODs for carboxylic acids, sugar phosphates and nucleotides spanned from 0.2 to 10 pmol for both analytical methods. Phosphoenolpyruvate, mesaconic acid, NADH and NADPH exhibited 5–10 times higher sensitivities with the PFPP method. NADP and ATP exhibited more than 4 times lower sensitivities in comparison with HILIC. However, this is not anticipated to pose any significant issues since their intracellular levels were about an order of magnitude above the LODs and within the linear range.

3.2. Absolute quantification of central metabolites of *M. extorquens AM1* using global ^{13}C-labeled I.Ss.

3.2.1. Method performance and matrix effects

To minimize the matrix effects and other experimental variations, in vivo synthesis of fully ^{13}C-labeled cell extracts were obtained by chemostat cultivation and a fixed amount was added as I.Ss. before cell extraction. The ^{13}C-labeled I.Ss. improved linearity for both the HILIC and PFPP separations with the correlation coefficients (R^2) of calibration curves for many of metabolites (68% of the metabolites analyzed by HILIC; 74% of the metabolites analyzed by PFPP) higher than 0.990 across a concentration range of two orders of magnitude (Table 1).

In order to validate the precision and accuracy of PFPP-MS/MS with global ^{13}C-labeled I.Ss., another group of 1, 5 and 25 μM of standard mixtures as quality controls (QCs) were spiked with the same amount of ^{13}C-labeled I.Ss. quantitated using calibration curves. The results indicated that for the most metabolites the precision ranged from 1% to 15% and accuracy from 0% to 20% (Fig. 3 and Supplemental Table 2). Although lower accuracy was observed for glycine at 28% and glutamine at 29% at 1 μM concentration, the

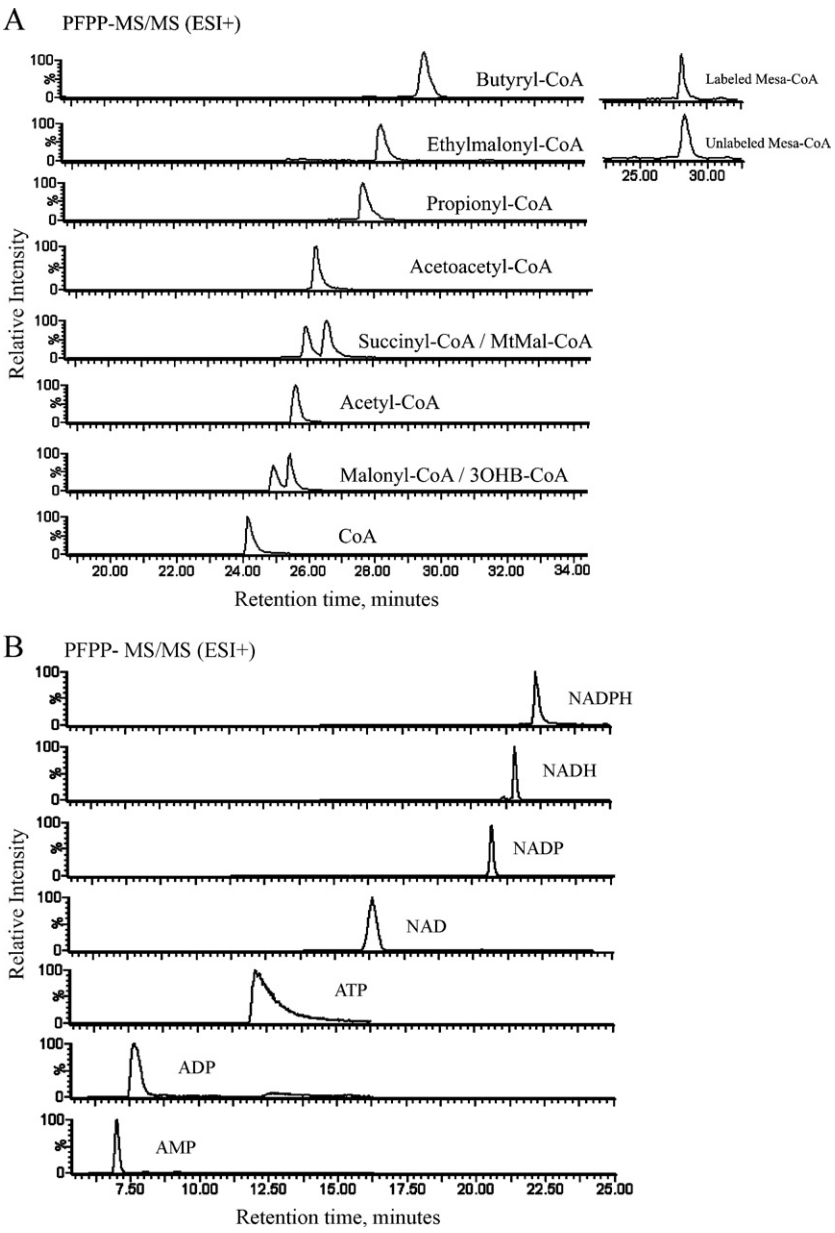

Fig. 2. The PFPP phase separation of acyl-CoAs and nucleotides. (A) Acyl-CoAs, right top 2 chromatograms were obtained from ethylamine cell extracts spiked with global ^{13}C-labeled I.Ss. The peaks likely correspond to mesaconyl-CoA (the lower peak was ^{12}C MRM, m/z 880 → 373; the upper peak was fully ^{13}C-labeled MRM, m/z 906 → 389). (B) Nucleotides. The standard mixtures were 5 μM of each compound. The abbreviations used are MtMal-CoA, methylmalonyl-CoA; 3OHB-CoA, 3-hydroxybutyryl-CoA; Mesa-CoA, mesaconyl-CoA.

levels in *M. extorquens* AM1 cell extracts were much higher than 1 μM.

We also used the QC samples to evaluate if only one I.S. was suitable to correct the variations of multiple metabolites from the same class. According to elution time, ^{13}C-labeled alanine, isoleucine and phenylalanine were selected as I.S. for the other amino acids, respectively; ^{13}C-labeled malate, succinate and mesaconic acid were selected for the other carboxylic acids and sugar phosphates, respectively; ^{13}C-labeled propionyl-CoA and ADP were selected for the other acyl-CoAs and nucleotides, respectively. Based on the comparison of accuracy, it was indicated that the use of a single I.S. for acyl-CoAs would be acceptable due to their close range of retention time and similar chemical structure (Supplemental Fig. 1). On the other hand, one I.S. of amino acids was less satisfactory for quantification of some other amino acids either at the low or high concentration of QCs (Supplemental Fig. 1). Similar results were also observed for the analysis of carboxylic acids.

As shown in Table 1, the LODs of standards spiked with ^{13}C-labeled I.Ss. were generally affected by the matrix effects by a factor of 1–4, depending on retention time, metabolite property and co-eluting compounds for either the HILIC or PFPP phase. A closer look at the LODs of amino acids indicated that values for lysine, glycine and glutamine increased more (a factor of more than 7.5) than the other amino acids on the PFPP phase. Lower sensitivity was likely caused by co-eluting compounds or analyte low mass (e.g. glycine) which increased the likelihood of interference from the mobile phase. This observation was consistent with the higher variability of accuracy described above. In comparison to the HILIC method, most of amino acids showed similar sensitivity on the PFPP phase.

The concentration levels of global fully ^{13}C-labeled I.Ss. were also determined from the calibration curves obtained by PFPP-MS/MS (Table 1). The lowest concentration of I.S. was determined for ethylmalonic acid at 0.5 μM. The highest concentration was

Table 1
HILIC–MS/MS and PFPP-MS/MS methods performance of standards and standards spiked with global ^{13}C-labeled I.Ss., and the list of concentrations of global fully ^{13}C-labeled I.Ss.

Metabolites	LOD standards (pmol, on column)		LOD standards spiked with global ^{13}C-labeled I.Ss. (pmol, on column)		R^2 of calibration curve (spiked with global ^{13}C-labeled I.Ss.)		Concentration (^{13}C-I.Ss. μM)
	HILIC	PFPP	HILIC	PFPP	HILIC	PFPP	
Amino acids							
Alanine	0.8	0.3	2.0	1.5	0.995	0.996	45.3
Aspartic acid	0.6	0.2	1.5	0.8	0.981	0.985	4.2
Glutamic acid	0.4	0.2	1.2	1.0	0.994	0.995	262.6
Glutamine	0.5	0.2	1.0	1.5	0.993	0.998	70.6
Glycine	7.0	0.5	15.0	5.0	0.992	0.988	8.5
Isoleucine	0.1	0.1	0.2	0.2	0.994	0.998	2.1
Leucine	0.1	0.06	0.2	0.1	0.994	0.997	9.9
Lysine	3.0	0.2	5.5	2.5	0.989	[a]	[a]
Phenylalanine	0.1	0.3	0.2	0.5	0.996	0.996	2.8
Proline	0.5	0.2	1.2	0.5	0.992	0.995	9.6
Serine	1.0	0.2	2.5	0.7	0.991	0.993	6.4
Threonine	0.5	0.3	1.2	1.4	0.995	0.991	20.6
Valine	0.5	0.5	1.0	1.2	0.995	0.998	9.3
Carboxylic acids							
3-Hydroxybutyric acid	3.0	2.5	2.0	3.0	0.992	0.990	0.9
α-Ketoglutaric acid	7.0	6.5	10.0	10.0	0.991	0.993	12.6
Citric acid	0.8	1.5	1.5	1.0	0.993	0.998	8.1
Ethylmalonic acid	1.5	0.5	3.5	1.5	0.990	0.991	0.5
Fumarate	3.0	4.5	2.5	7.5	0.986	0.992	5.1
Glyceric acid	1.0	2.0	2.0	4.0	0.983	0.985	3.2
Glycolic acid	2.5	1.5	3.0	5.0	0.985	0.990	2.5
Glyoxylic acid	5.0	6.0	40.0	50.0	[b]	[b]	[b]
Isocitric acid	0.8	2.0	1.5	3.0	[c]	[c]	[c]
Malate	0.5	1.0	1.5	3.0	0.993	0.993	7.8
Mesaconic acid	7.5	0.9	12.5	2.5	0.992	0.999	1.4
Methylsuccinic acid	1.5	0.8	3.5	1.5	0.988	0.991	1.5
Methylmalonic acid	10.0	5.0	15.0	5.0	[c]	[c]	[c]
Phosphoenolpyruvate	3.5	0.5	2.5	1.5	0.987	0.992	6.3
Pyruvate	5.0	7.5	7.5	9.0	0.989	0.988	5.9
Succinate	10.0	10.0	15.0	12.0	0.985	0.991	11.6
Nucleotides							
ADP	2.0	1.5	3.0	3.5	0.990	0.994	12.3
AMP	1.0	0.4	2.0	1.0	0.991	0.992	5.1
ATP	1.5	7.0	4.0	15.0	0.992	0.992	35.0
NAD	0.5	1.0	1.2	1.0	0.995	0.995	15.7
NADH	2.0	0.2	3.5	0.5	[d]	[d]	[d]
NADP	0.5	2.0	1.5	4.5	0.995	0.996	4.6
NADPH	4.0	0.8	8.0	2.0	[d]	[d]	[d]
Acyl-CoAs							
3-Hydroxybutyryl-CoA	[e]	0.7	[e]	2.0	[e]	0.991	3.2
Acetoacetyl-CoA	[e]	1.5	[e]	4.0	[e]	[d]	[d]
Acetyl-CoA	[e]	1.0	e	2.5	[e]	0.995	5.1
Butyryl-CoA	[e]	1.0	e	3.0	[e]	0.998	1.6
CoA	[e]	0.5	e	1.5	[e]	0.996	8.6
Ethylmalonyl-CoA	[e]	1.0	e	2.5	[e]	0.996	1.2
Malonyl-CoA	[e]	1.5	e	3.0	[e]	0.993	[d]
Mesaconyl-CoA	[e,f]	[f]	[e,f]	[f]	[e,f]	[f]	[f]
Methylmalonyl-CoA	[e]	0.5	[e]	1.5	[e]	0.992	3.0
Propionyl-CoA	[e]	0.5	[e]	2.0	[e]	0.991	3.2
Succinyl-CoA	[e]	1.0	[e]	2.5	[e]	0.990	0.8
Sugar-phosphates							
6-phosphogluconic acid	3.5	2.0	4.0	5.0	0.985	0.989	5.7
Fructose 6-phosphate	1.0	0.5	2.5	2.0	0.992	0.995	15.6[g]
Glucose-6-phosphate	1.0	0.5	2.5	2.0	0.992	0.995	15.6[g]

[a] Not reproducible by PFPP-MS/MS.
[b] Poor MS signal sensitivity.
[c] Trace amounts in the normal cell extracts.
[d] Poor stability during the cell extraction.
[e] Not analyzed by HILIC–MS/MS.
[f] Mesaconyl-CoA standard was not available by commercial purchase.
[g] The value was the combined concentrations of fructose-6-phosphate and glucose-6-phosphate.

determined for glutamic acid at 262.6 μM. The concentrations of glutamic acid and glutamine were above the upper boundary (50 μM) of the initial calibration curve linear range. For this case, the calibration curves were extended to 400 μM and a linear response was observed from 2.5 to 400 μM (A_{un}:A_{ful} ratio was from 0.017 to 1.324) for glutamic acid, and 1.0 to 200μM (A_{un}:A_{ful} ratio was from 0.02 to 2.48) for glutamine.

3.2.2. Effect of incomplete labeling I.Ss.

It is possible that some intracellular metabolites might be unlabeled or partially labeled due to the assimilation of CO_2 during the growth of the initial batch phase, low turnover rate for some macromolecules and lesser extent ^{12}C contamination of the uniformly ^{13}C-labeled substrate (^{12}C-MeOH < 1%). Therefore, an estimation of the percentage of metabolites that were fully labeled was carried

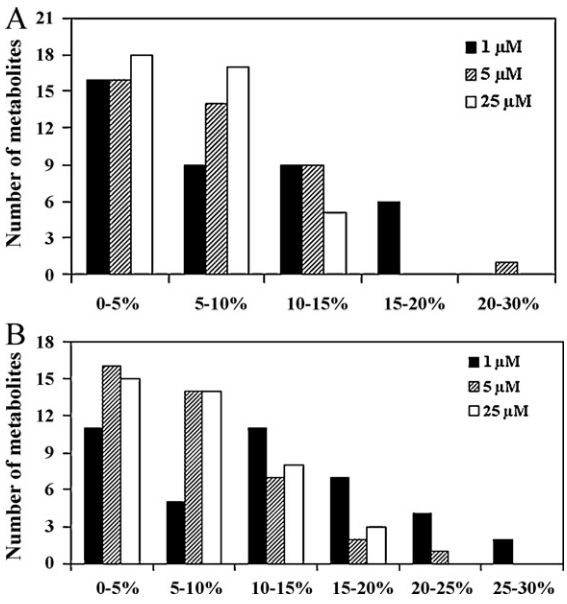

Fig. 3. Summary of validation results of precision and accuracy (A) Histogram of the distribution of precision of 40 metabolites in the QCs determined by PFPP-MS/MS with global ^{13}C-labeled I.Ss. (B) Histogram of the distribution of accuracy of 40 metabolites in the QCs determined by PFPP-MS/MS with global ^{13}C-labeled I.Ss. Accuracy (%) was defined as measured value minus real value divided by real value. The data was presented as the mean of three replications at each concentration.

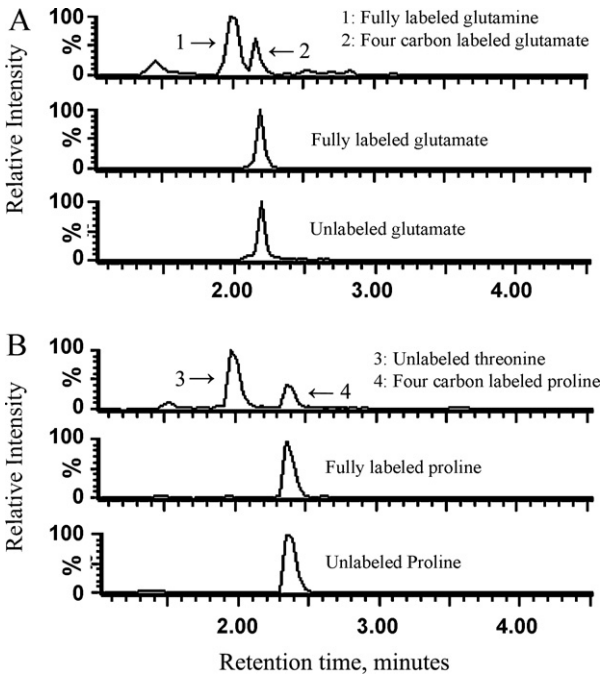

Fig. 4. Example chromatograms resolving the contamination of partially labeled metabolites on the PFPP column. The chromatograms were obtained from cell extracts of *M. extorquens* AM1 spiked with global ^{13}C-labeled I.Ss. (A) Glutamine and glutamate and (B) threonine and proline.

out on both LC–MS and GC/MS at the initial experimental phase. For most of the metabolites, the unlabeled peak areas were less than 1% compared to the fully ^{13}C-labeled peak areas, indicating the unlabeled fraction in the ^{13}C-cell extracts that could interfere with quantification was negligible in the analysis of real samples (data not shown). For 3-hydroxybutyric acid, succinyl-CoA and glycolic acid (metabolites involved in the PHB cycle, TCA cycle), the unlabeled percentage was over 60%, thus the I.S. contribution to the analyte amounts should be corrected in the real samples.

Partial labeling of metabolites was present in all the targeted metabolites. This might interfere with the quantification (for example: 4-carbon labeled glutamic acid (*m/z* 152–88) could contribute to the abundance of 5-carbon labeled glutamine (*m/z* 152–88); four carbon labeled proline (*m/z* 120–74) could contribute the abundance of unlabeled threonine (*m/z* 120–74)) (Fig. 4). These potential interferences were successfully resolved on the PFPP column.

3.3. Relative abundance and absolute concentration of central metabolites of M. extorquens AM1 grown on two different substrates

To assess the methodology, we analyzed central metabolites of *M. extorquens* AM1 grown on either ethylamine (C2) or succinate (C4), and compared to previously published results obtained using a combined method of LC–MS/MS and GC × GC–TOF-MS (Fig. 5) [18]. The relative abundance (ratio) of metabolites in C2 culture versus C4 culture was determined by comparing normalized peak areas (Fig. 5). The relative standard deviation (RSD) of ratios ranged from 12% to 34% with an average of 21%, which was within acceptable variation limits considering the range of typical biological variation. The PFPP-MS/MS approach provided consistent trends for most of the metabolites involved in the serine cycle, EMC pathway, TCA cycle and nucleotide energy, as compared to the previous results [18]. Ratios outside the 1.00 ± 0.20 range indicated significant differences of absolute concentration for metabolite (FDR < 0.05).

As shown in Table 2, the developed PFPP-MS/MS method with global ^{13}C-labeled I.Ss. was further applied for obtaining absolute concentration of primary metabolites. The absolute concentrations calculated using the calibration curves were normalized to cell biomass. The energy charge (EC) reflects the energy state of a cell and is an indication of the quality of the sample. Here EC was 0.79 ± 0.03 for the succinate culture and 0.80 ± 0.04 for the ethylamine culture, matching well with the expected value for growing *M. extorquens* AM1 cells [34]. The lowest concentration detected was 0.051 μmol/g for ethylmalonic acid in the succinate culture, and the highest concentration was 36.83 μmol/g for glutamic acid in the succinate culture. The RSD ranged from 5.3% (phenylalanine in the succinate culture) up to 27.3% (3-hydroxybutyric acid in the succinate culture). *M. extorquens* AM1 uses specific pathways for C2 and C4 assimilation that are essential for growth on C2 compounds (the ethylmalonyl-CoA or EMC pathway) and for growth of C4 compounds (TCA cycle) [35,29]. The changes of pool size of central metabolites were clearly observed for some amino acids, carboxylic acids and acyl-CoAs involved in these two pathways and related pathways. The amounts of citric acid, α-ketoglutaric acid and fumarate, involved in the TCA cycle, were found to be 2.5–5-fold higher in the succinate culture. Ethylmalonic acid, mesaconic acid and important acyl-CoAs such as ethylmalonyl-CoA and mesaconyl-CoA involved in the EMC pathway were found to increase around 2–3-fold in the ethylamine samples. In addition, about 2-fold increases of serine suggests activation of a separate pathway involved in use of C2 compounds in the ethylamine culture [36].

4. Discussion

Metabolomics is becoming a powerful tool for identifying metabolic pathways and metabolic flux in a variety of applications. Most protocols for measuring a broad range of compounds of interest in metabolic pathways are multimodal, involving multiple extraction protocols, multiple columns, and/or multiple instruments [9,17,37]. In addition, with a broad range of compounds detected, it is common to report only fold-changes rather than

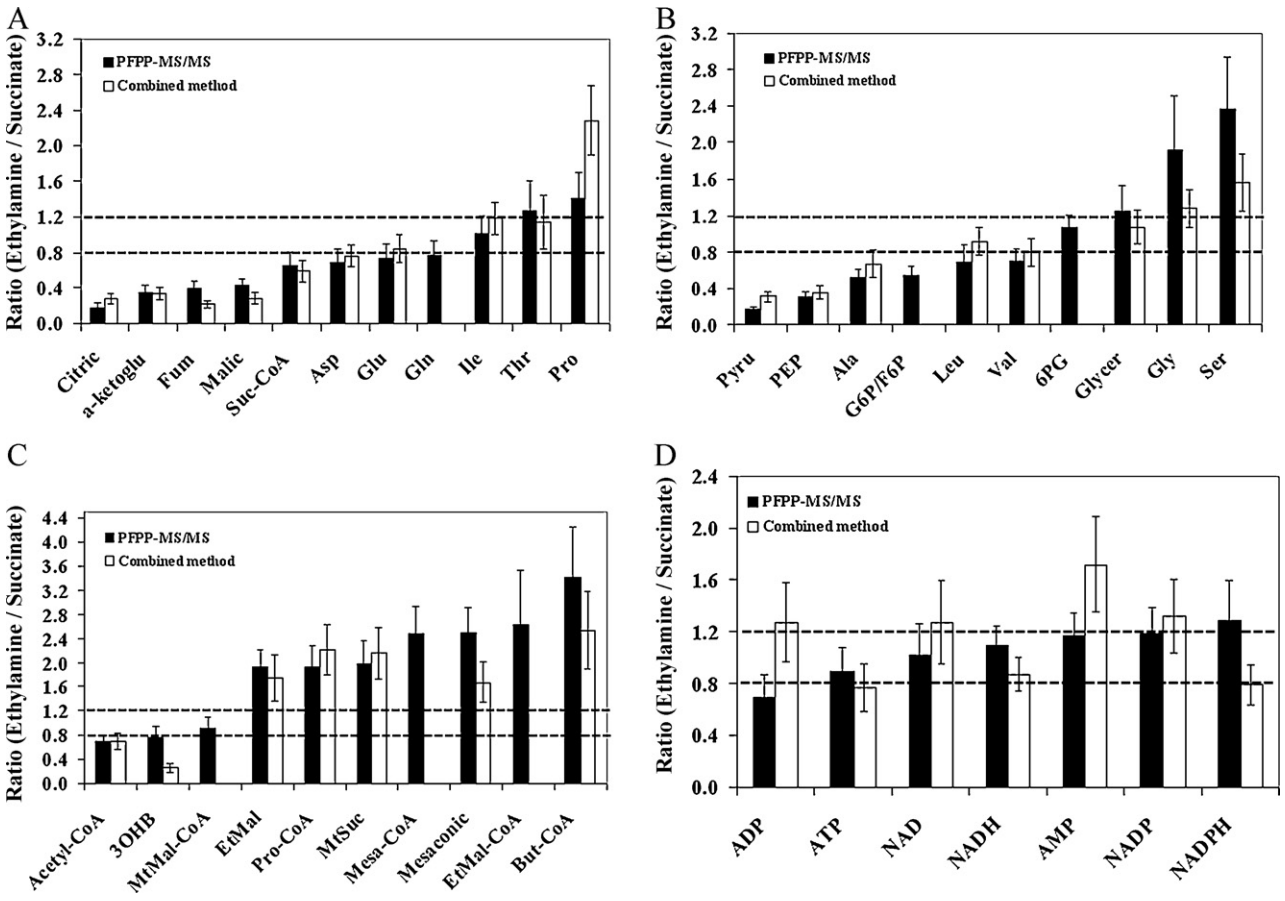

Fig. 5. Comparison of abundance ratios of metabolites in *M. extorquens* AM1 grown on two different substrates analyzed by the PFPP-MS/MS method and the published combined method [18]. Metabolites not measured in the combined method are not displayed. The ratios outside of the two dashed lines represent a significant difference between the two growth conditions (FDR < 0.05) except for the succinyl-CoA (FDR = 0.092). (A) TCA cycle and related amino acid biosynthesis, (B) serine cycle and related pathways, (C) EMC pathway and (D) nucleotides. The abbreviations used are α-ketoglu, α-ketoglutaric acid; Pyru, pyruvate; Fum, fumaric acid; Glycer, glyceric acid; 3OHB, 3-hydroxybutyric acid; EtMal, ethylmalonic acid; MtSuc, methylsuccinic acid; PEP, phosphoenolpyruvate; Suc-CoA, succinyl-CoA; Pro-CoA, propionyl-CoA; But-CoA, butyryl-CoA; Mesa-CoA, mesaconyl-CoA; EtMal-CoA, ethylmalonyl-CoA; MtMal-CoA, methylmalonyl-CoA; 6PG, 6-phosphogluconic acid; G6P/F6P, glucose-6-phosphate/fructose 6-phosphate.

absolute concentrations [38,39]. Although the ability to assess fold-changes is useful in some areas, generally absolute quantitation is preferred, and is especially important for kinetic modeling and for flux analysis [4–6]. The level of effort required and limited availability of internal standards for quantitative measurement of a broad range of metabolites have decreased the broad applicability of metabolomics for studies of metabolism. Approaches to streamline quantitative metabolomic analysis are needed, to facilitate adoption of metabolomics as a standard tool in metabolic studies. In this study, we used a single PFPP column in an LC–MS/MS approach combined with global ^{13}C-labeled standards from cell extracts to develop a simplified analytical protocol for quantitative metabolomics. Our focus was not only to set up an analytical method to detect analytes of standards as many as possible, but also to determine absolute concentration in bacterial cells extracts.

When we compared the PFPP column to the HILIC column, a clear advantage of the former was the ability to distinguish the metabolites with similar physicochemical characterics. A number of isomers (e.g. citrate/isocitrate; succinyl-CoA/methylmalonyl-CoA; methylsuccinic acid/ethylmalonic acid) or similar fragment ion metabolites (e.g. malate/fumarate; butyryl-CoA/acetyl-CoA) are involved in related metabolic networks, and thus their parallel quantification plays an important role in elucidating biological pathways. Since they are difficult to distinguish directly by mass spectrometry, their chromatographic separation becomes particularly important. Under the HILIC conditions used here, many critical pairs which were known to be of specific importance for *M. extorquens* AM1 metabolism were partially or totally overlapped in the chromatogram, creating the possibility of overestimating the amounts of one member of each pair and resulting in the possible misinterpretation of metabolic reactions. Similar suboptimal performance for these compounds on the HILIC phase was also observed in other studies, suggesting that the HILIC mode does not separate well some isomers or metabolites with similar structure due to their hydrophilic similarity [14,15,40]. In comparison to a HILIC phase, the majority of primary metabolites, including 10 critical pairs and short chain acyl-CoAs, were successfully resolved in the cell extracts by the multiple separation mechanisms of the PFPP column [21–23]. This separation advantage provided an important basis in the analysis of central metabolic status in *M. extorquens* AM1 and valuable insight for comparing different metabolic modes in a single PFPP-MS/MS approach. In addition, it should be noted that compared to the HILIC phase, some limitation can be inferred from practical applications of the PFPP phase. For example, the organic cell extracts or the eluted fractions from solid-phase extraction cartridge must be evaporated and redissolved in acidified water in order to obtain a reproducible interaction with reverse phase chromatographic system, a step that may result in decomposition or interconversion of labile metabolites.

The current study indicated that the occurrence of matrix effects was indeed significant for the majority of metabolites either on HILIC–MS/MS or PFPP-MS/MS, thus quantification required effi-

Table 2
List of concentrations of central metabolites determined by PFPP-MS/MS with global ^{13}C-labeled I.Ss. *M. extorquens* AM1 was grown on either ethylamine (C2) or succinate (C4). The bold metabolites represent a significant difference between the two growth conditions (FDR < 0.05).

Metabolites	Absolute concentration[a]	
	Succinate (μmol/g cdw)	Ethylamine (μmol/g cdw)
TCA cycle and related amino acids		
α-Ketoglutaric acid	2.12 ± 0.27	0.73 ± 0.14
Aspartic acid	0.35 ± 0.09	0.24 ± 0.04
Citric acid	1.43 ± 0.32	0.25 ± 0.05
Fumarate	0.55 ± 0.06	0.23 ± 0.02
Glutamic acid	36.84 ± 6.44	29.45 ± 4.49
Glutamine	12.51 ± 2.01	9.04 ± 1.75
Isoleucine	0.29 ± 0.04	0.28 ± 0.05
Malate	1.69 ± 0.32	0.69 ± 0.09
Proline	1.13 ± 0.20	1.62 ± 0.24
Succinate	[b]	0.44 ± 0.05
Succinyl-CoA	0.47 ± 0.07	0.31 ± 0.06
Threonine	1.20 ± 0.21	1.53 ± 0.37
EMC pathway		
3-Hydroxybutyryl-CoA	0.15 ± 0.02	0.35 ± 0.06
3-Hydroxybutyric acid	0.11 ± 0.03	0.083 ± 0.014
Acetyl-CoA	1.44 ± 0.16	0.97 ± 0.15
Butyryl-CoA	0.11 ± 0.02	0.38 ± 0.07
Ethylmalonic acid	0.051 ± 0.008	0.098 ± 0.010
Ethylmalonyl-CoA	0.072 ± 0.018	0.19 ± 0.05
Mesaconic acid	0.062 ± 0.009	0.16 ± 0.02
Mesaconyl-CoA[c]	0.083 ± 0.010	0.21 ± 0.04
Methylmalonyl-CoA	0.21 ± 0.03	0.19 ± 0.03
Methylsuccinic acid	0.058 ± 0.009	0.11 ± 0.01
Propionyl-CoA	0.29 ± 0.05	0.56 ± 0.07
Serine Cycle and related pathways		
6PG[d]	0.21 ± 0.03	0.19 ± 0.01
Alanine	2.03 ± 0.23	1.08 ± 0.15
G6P/F6P[d]	1.79 ± 0.26	0.90 ± 0.06
Glyceric acid	0.33 ± 0.05	0.41 ± 0.07
Glycine	0.82 ± 0.19	1.56 ± 0.38
Leucine	1.03 ± 0.22	0.69 ± 0.12
Phosphoenolpyruvate	1.43 ± 0.23	0.45 ± 0.09
Pyruvate	2.12 ± 0.25	0.36 ± 0.06
Serine	0.62 ± 0.14	1.42 ± 0.36
Valine	1.22 ± 0.26	0.85 ± 0.12
Energy and reduced		
ADP	2.03 ± 0.40	1.39 ± 0.27
AMP	0.38 ± 0.03	0.40 ± 0.05
ATP	4.21 ± 0.71	3.58 ± 0.52
NAD	1.66 ± 0.26	1.82 ± 0.23
NADH[e]	0.22 ± 0.04	0.21 ± 0.04
NADP	0.33 ± 0.03	0.36 ± 0.06
NADPH[e]	0.49 ± 0.10	0.64 ± 0.11
$EC = (ATP + 0.5\ ADP)/(ATP + ADP + AMP)$	0.79 ± 0.03	0.80 ± 0.04
Other pathways		
CoA	0.84 ± 0.14	0.61 ± 0.09
Glycolic acid	0.35 ± 0.08	0.56 ± 0.14
Phenylalanine	0.38 ± 0.02	0.43 ± 0.05

[a] Mean of three independent biological experiments.
[b] Not available as succinate was present as a carbon source.
[c] Mesaconyl-CoA was estimated by using propionyl-CoA as I.S.
[d] 6PG, 6-phosphogluconic acid; G6P/F6P, glucose-6-phosphate/fructose 6-phosphate.
[e] NADH and NADPH were not stability during the cell extraction.

cient compensation strategies such as the use of the standard addition method or ^{13}C-labeled I.Ss. While the standard addition method works for an individual analyte quantitation, it is not very useful for metabolomic studies as it adds complexity to the workflow and may significantly modify the matrix [40]. Furthermore, we evaluated whether utilization of a single ^{13}C-labeled I.S. could substitute for the use of global ^{13}C-labeled I.Ss.. Although some of the metabolites were acceptable to be quantified by using one selected I.S., unsatisfactory accuracy, especially for amino acids and carboxylic acids, was observed at low and high concentration of QCs (Supplemental Fig. 1). A possible explanation is that various metabolites can be subjected to different matrix effects, which has been classified into three distinct cases on the LC–MS/MS platform in Ref. [41]. Our study demonstrated that the addition of one or two I.Ss. was not satisfactory for correction of matrix effects for all the other metabolites. This conclusion contrasts to the observation obtained using GC/MS instrumentation, which suggested that two I.Ss. were adequate for correcting matrix effects of other metabolites [42]. For absolute quantification using current method, the application of global ^{13}C-labeled I.Ss. was needed to improve linearity of calibration curves. Since the global I.Ss. were generated in the most relevant way to the studied cell culture in terms of metabolite levels and matrix effects, the accuracy of quantification is improved (Supplemental Fig. 1). The average RSD for the real samples was 17% in the succinate culture and 16% in the ethylamine culture. This reproducible result allowed the detection of small fold changes of intermediates between two growth conditions. This shows that the choice of I.Ss. should take into consideration of the sample preparation, separation method and detector. It is worth noting that although different chromatographic conditions (PFPP, HILIC, RPLC, GC or GC × GC) or instrumental combinations (LC–MS/MS, GC/MS or GC × GC–TOF-MS) could produce different matrix effects and signal to noise ratios, these alterations do not significantly interfere with the comparison of relative abundance under the two growth conditions (Fig. 5). A similar observation was made previously, demonstrating that matrix effects could be negligible for monitoring relative changes in biological sample [43]. In addition, the consistency of the obtained ratio between the PFPP-MS/MS method and the combined method further suggested that a single PFPP-MS/MS method has the same capability of obtaining comprehensive metabolite profile comparison as the combined method.

By the PFPP-MS/MS approach, 42 of 50 metabolites could be reliably measured in the cell extracts. The remaining 8 metabolites included 1 amino acid (lysine), 2 acyl-CoAs (malonyl-CoA and acetoacetyl-CoA), 2 redox cofactors (NADPH and NADH) and 3 carboxylic acids (glyoxylate, isocitric acid and methylmalonic acid). The pool sizes of NADH and NADPH could not be accurately measured because they are sensitive to extraction temperature and solvent pH. Acetoacetyl-CoA and malonyl-CoA may have decomposed during the extraction. However, these problems were not related to the PFPP separation with addition of global ^{13}C-labeled I.Ss., and could be reduced by using alternative extraction procedures [34,44]. Isocitric acid and methylmalonic acid could not be well detected because of their trace concentrations in *M. extorquens* AM1 cells, but could be detected in concentrated cell extracts. Glyoxylate could not be detected because of large reduction of signal sensitivity by matrix effects. The measurement of glyoxylate was also the biggest challenge on the HILIC mode due to ion suppression and on the regular GC platform due to the occurrence of compounds that coeluted and presented common fragment ions [15,41]. Lysine could not be reproducibly detected because it eluted early past the void time. Overall, in comparison to the combined method of LC–MS/MS and GC × GC–TOF-MS, most of the targeted primary metabolites could be measured on a single PFPP-MS/MS approach, covering broad physicochemical properties from highly polar to moderately polar compounds. Only two metabolites (lysine and glyoxylate) that could not be ideally measured were related to the PFPP chromatography or MS sensitivity reduction. Nevertheless, the time consumed on the sample preparation and running was significantly reduced, and absolute quantification of real samples and reproducibility of the data were significantly improved for central metabolites by the single PFPP-MS/MS analysis with global ^{13}C-labeled I.Ss. If metabolites that are poorly measured must be detected to answer a specific biological question, a GC × GC-based method could be used as a complementary tool in connection with data deconvolution for the combined ^{12}C and ^{13}C spectra [32,45].

We successfully observed clear differences in the pool size of many intermediates between cultures of *M. extorquens* AM1 grown on either C2 or C4 compounds. The increase of methylsuccinic acid, ethylmalonic acid, propionyl-CoA and ethylmalonyl-CoA etc. in the C2 culture were consistent with previous results [18,35,46]. The increase of serine in the C2 culture further supported another recent finding obtained by microarray and mutant analysis that a portion of the serine cycle for C1 assimilation could participate in C2 assimilation [36]. These important changes reflected highly differing metabolic fluxes in metabolic pathways under different carbon substrate conditions. The more accurate quantification obtained by the streamlined analytical method presented here forms an important basis for flux measurements and can be used for metabolism modeling in *M. extorquens* AM1 in future studies.

5. Conclusion

An approach involving PFPP-MS/MS with culture-derived global ^{13}C-labeled I.Ss. was developed and applied to determine a quantitative metabolome in central metabolism of *M. extorquens* AM1 grown on different carbon sources. In comparison with the single HILIC mode, many primary metabolites, especially important metabolic pairs such as isomers (e.g. methylsuccinic acid/ethylmalonic acid), and similar structure metabolites (e.g. malate/fumarate) and acyl-CoAs (e.g. succinyl-CoA/methylmalonyl-CoA) were simultaneously resolved by PFPP chromatography. In comparison with a previously combined HILIC–MS/MS, RPLC–MS/MS and GC × GC–TOF-MS method, the use of a single PFPP column presented consistent relative abundance changes in C2- versus C4-grown cultures, while it has the capability of analysis of a similar number of metabolites. The validation of the analysis demonstrated that addition of global ^{13}C-labeled I.Ss. was beneficial to obtain better accurate quantification due to matrix effects and other system variations. The presented methodology provides a good streamlined workflow for comprehensive metabolite profiling and metabolome quantitation, which can provide fundamental data in the analysis of central metabolic status in bacteria.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.09.055.

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