analytical chemistry

ACS AuthorChoice

Article

Cite This: Anal. Chem. 2018, 90, 4470−4477

pubs.acs.org/ac

Quantitative Isotope-Dilution High-Resolution-Mass-Spectrometry Analysis of Multiple Intracellular Metabolites in *Clostridium autoethanogenum* with Uniformly ^{13}C-Labeled Standards Derived from Spirulina

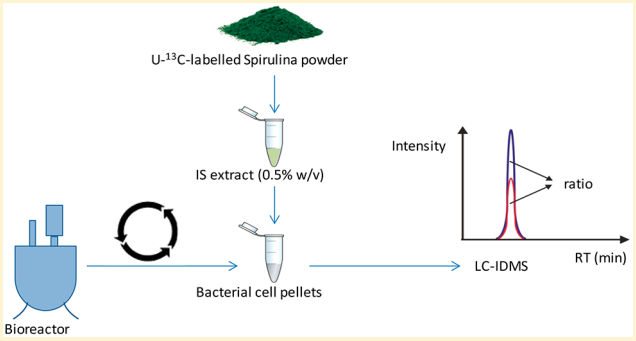
Sarah Schatschneider,[⊥,†,§] Salah Abdelrazig,[⊥,†] Laudina Safo,[†] Anne M. Henstra,[‡] Thomas Millat,[‡] Dong-Hyun Kim,[†] Klaus Winzer,[‡] Nigel P. Minton,[‡] and David A. Barrett*[,†]

[†]Centre for Analytical Bioscience, School of Pharmacy, University of Nottingham, Nottingham NG7 2RD, U.K.

[‡]Clostridia Research Group, SBRC-Nottingham, a BBSRC/EPSRC Synthetic Biology Research Centre, School of Life Sciences, University of Nottingham, Nottingham NG7 2RD, U.K.

Supporting Information

ABSTRACT: We have investigated the applicability of commercially available lyophilized spirulina (*Arthrospira platensis*), a microorganism uniformly labeled with ^{13}C, as a readily accessible source of multiple ^{13}C-labeled metabolites suitable as internal standards for the quantitative determination of intracellular bacterial metabolites. Metabolites of interest were analyzed by hydrophilic-interaction liquid chromatography coupled with high-resolution mass spectrometry. Multiple internal standards obtained from uniformly (U)-^{13}C-labeled extracts from spirulina were used to enable isotope-dilution mass spectrometry (IDMS) in the identification and quantification of intracellular metabolites. Extraction of the intracellular metabolites of *Clostridium autoethanogenum* using 2:1:1 chloroform/methanol/water was found to be the optimal method in comparison with freeze−thaw, homogenization, and sonication methods. The limits of quantification were ≤1 μM with excellent linearity for all of the calibration curves ($R^2 \geq 0.99$) for 74 metabolites. The precision and accuracy were found to be within relative standard deviations (RSDs) of 15% for 49 of the metabolites and within RSDs of 20% for all of the metabolites. The method was applied to study the effects of feeding different levels of carbon monoxide (as a carbon source) on the central metabolism and Wood−Ljungdahl pathway of *C. autoethanogenum* grown in continuous culture over 35 days. Using LC-IDMS with U-^{13}C spirulina allowed the successful quantification of 52 metabolites in the samples, including amino acids, carboxylic acids, sugar phosphates, purines, and pyrimidines. The method provided absolute quantitative data on intracellular metabolites that was suitable for computational modeling to understand and optimize the *C. autoethanogenum* metabolic pathways active in gas fermentation.



Quantitative biology aims to explain the function of an entire biological system, and intracellular-metabolite analysis is an important aspect of this approach because it reveals functional information about the biochemical and physiological states of cells.[1,2] Metabolomics can provide "global" information on metabolites, but frequently this is not quantitative in nature (for example, by not providing precise concentrations) and is thus difficult to apply in accurate modelings of intracellular processes required in systems biology and metabolic engineering. LC-MS methods have previously been reported for the simultaneous quantification of different classes of intracellular metabolites in microorganisms using mainly hydrophilic interaction liquid chromatography (HILIC)[3,4] and ion-pair reversed-phase chromatography.[5,6] Although such LC-MS methods produce comprehensive data, there are significant limitations in achieving the absolute quantification of multiple metabolites, with ion enhancement or ion suppression in LC-MS analyses caused by matrix effects, the degradation of metabolites during sample preparation, and unexpected variation in instrument responses. These factors all result in the potentially biased quantification of measured metabolite concentrations.

True quantitative analyses can be achieved using synthesized isotopic internal standards (IS) for a small number of metabolites simultaneously.[7] Isotope-dilution mass spectrometry (IDMS) is the most reliable technique for the generation of accurate, MS-derived quantitative metabolite data because this approach can correct for most aspects of analytical biases.[8,9] Historically, the application of isotope dilution utilized

Received: November 17, 2017
Accepted: March 13, 2018
Published: March 13, 2018

DOI: 10.1021/acs.analchem.7b04758
Anal. Chem. 2018, 90, 4470−4477

radioactive tracers for the quantification of a single or a small set of analytes. Now, this has been replaced by the use of nonradioactive stable isotopes.[8] However, as the number of metabolites monitored has increased in recent years, the availability of matching isotopic standards for analytes has become increasingly difficult to achieve. Most isotopically labeled compounds are difficult to synthesize and are not available commercially. The biosynthesis of multiple uniformly ^{13}C-labeled (U-^{13}C) IS for LC-IDMS is an alternative approach that attempts to bypass the synthetic bottleneck by cultivating organisms on ^{13}C-labeled substrates as the sole carbon sources. For example, *Escherichia coli* or yeast grown on ^{13}C-glucose or ^{15}N-labeled substrate sources, have been used to generate labeled IS of metabolites for LC-IDMS in targeted metabolomics or metabolic-flux analyses[10−13] or for MS-method improvements.[14,15] However, the biosynthesis of multiple U-^{13}C IS is time-consuming and requires an expensive U-^{13}C carbon source, access to a microbiological laboratory, and the associated skills. Commercially available U-^{13}C spirulina (*Arthrospira platensis*) has the potential to be used as a cheap and readily accessible source of multiple U-^{13}C IS suitable for quantitative LC-IDMS of bacterial metabolites.

Clostridium autoethanogenum is a Gram-positive bacterial species of biotechnological interest as it can fix $CO/CO_2/H_2$ to produce industrially useful chemicals, such as ethanol and 2,3-butanediol.[16−18] The yields of these chemicals are low in the wild-type organism, and metabolic engineering and the optimization of bioreactor conditions are required for efficient production. Specifically, our work required the generation of accurate and quantitative measurements of key intracellular metabolites of *C. autoethanogenum* to deliver important information for computational metabolic modeling.

It is difficult to biosynthesize U-^{13}C-labeled internal standards in *C. autoethanogenum* because of the challenges with growth on CO_2 and CO as carbon sources. Therefore, in this study, we evaluated a new method for LC-IDMS analysis using purchasable U-^{13}C-spirulina material as a novel source of multiple IS for quantitative metabolomics. In previous studies, ^{15}N- or ^{13}C-labeled spirulina has mainly been used as a feedstock for the stable-isotope labeling of mammals[19−21] and has not yet been explored for analytical applications. Spirulina is supplied as a ready-to-use powder; thus, there is no need for the cultivation of microorganisms to generate fully labeled IS. The developed method was applied to the simultaneous quantification of 74 key intracellular metabolites in continuous cultures of gas-fermentation extracts of *C. autoethanogenum*.

EXPERIMENTAL SECTION

Chemicals. HPLC-grade ammonium carbonate and all of the analytical standards were purchased from Sigma-Aldrich (Gillingham, U.K.) unless otherwise noted. Uniformly ^{13}C-labeled spirulina (U-^{13}C, 97%) and ^{12}C spirulina lyophilized-cell powders were obtained from CK Isotopes (Ibstock, U.K.). MS-grade acetonitrile and methanol were purchased from VWR (Langenfeld, Germany). A set of 74 metabolites representing the key intracellular metabolic pathways of *C. autoethanogenum* was selected to monitor the intracellular metabolic changes. Individual stock solutions of 100 mM ^{12}C authentic standards of these metabolites were used to prepare three different 1 mM standard mixtures in 50% (v/v) methanol/water.

LC-IDMS Analysis. LC-IDMS was performed using an Accela or Dionex UHPLC system (Thermo Fisher Scientific, Waltham, MA) coupled to a high-resolution orbital-trap mass spectrometer (Exactive or Q-Exactive, Thermo Fisher Scientific, Waltham, MA). A ZIC-*p*HILIC column (4.6 × 150 mm, 5 μm particle size, Merck Sequant, Watford, U.K.), maintained at 45 °C with a flow rate of 300 μL/min, was used for the chromatographic separation. Mobile phase A was composed of 20 mM ammonium carbonate in water (pH 9.1), and mobile phase B was composed of acetonitrile. The gradient started with 20% A and increased to 95% A over 8 min. This was followed by equilibration to give a 15 min run time. The injection volume was 5 μL, and samples were maintained at 4 °C during the analysis. MS was performed in simultaneous ESI + and ESI− full-scan modes with spray voltages of 4.5 (ESI+) and 3.5 kV (ESI−) and capillary voltages of 40 (ESI+) and −30 V (ESI−). In both modes, the sheath-, auxiliary-, and sweep-gas flow rates were 40:5:1 arb unit, respectively, and the capillary and heater temperatures were 275 and 150 °C, respectively. Data were acquired with an automatic gain control of 1×10^6 and a resolution of 140 000 from m/z 70 to 1050.

Extraction and Characterization of U-^{13}C-Labeled Internal Standards (IS) from Spirulina. U-^{13}C-labeled intracellular metabolites were extracted from spirulina (0.5%, w/v) with methanol (−20 °C), vortex-mixed for 30 s, stored overnight at −20 °C, and centrifuged (10 000*g*, 5 min) to remove the cell debris. The supernatant was filtered through a 3 kDa cutoff filter (Amicon Ultra, Merck, Watford, U.K.) and spiked with 20 μM 2-fluoro-2-deoxyuridine (fluorinated-^{12}C IS). The spirulina extract was stored at −80 °C for use as U-^{13}C-labeled multiple IS for LC-IDMS. Untargeted analysis was performed to check the coverage of metabolites in both organisms. Extracts of spirulina and *C. autoethanogenum* and blanks were prepared in biological triplicates and analyzed with QC samples and 5 mixtures of 250 authentic standards in a single analytical run. XCMS,[22] mzMatch,[23] and IDEOM software[24] were then used for untargeted data analysis, as detailed by Kim et al.[25] Metabolite identification was carried out by matching the accurate masses and RTs of the chromatographic peaks with those of the authentic standards (level 1 identification) according to the metabolomics standards initiative.[26] Level 2 identification was carried out for the rest of the metabolites when standards were not available, and therefore accurate masses and predicted RTs were used (see Supporting Information Table S-1).

Validation and Quality Control of LC-IDMS for the Quantification of *C. autoethanogenum* Metabolites. Calibration curves, validations, and metabolite quantifications were generated using TraceFinder 3.1 software (Thermo Fisher Scientific, Waltham, MA). Thirteen calibration standards in the range of 1 nM to 200 μM were prepared in water from each standard mixture and spiked with 1:1 (v/v) 0.5% spirulina extracts (U-^{13}C IS) and analyzed with LC-IDMS (n = 5). The validity of the analytical method was assessed by measuring the linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, and repeatability of each standard according to the FDA guidelines for bioanalysis.[27] Signal-to-noise ratios of 3:1 and 10:1 were used to estimate the LODs and LOQs, respectively, whereas the correlation coefficient (R^2) was used as a measure of linearity. The precision was reported as the relative standard deviation (RSD) of the standard response, and the accuracy was determined as the percentage of the measured concentration of the analyte compared with the true value. For the metabolite quantification, all of the calibration standards, the blank, and the

DOI: 10.1021/acs.analchem.7b04758
Anal. Chem. 2018, 90, 4470−4477

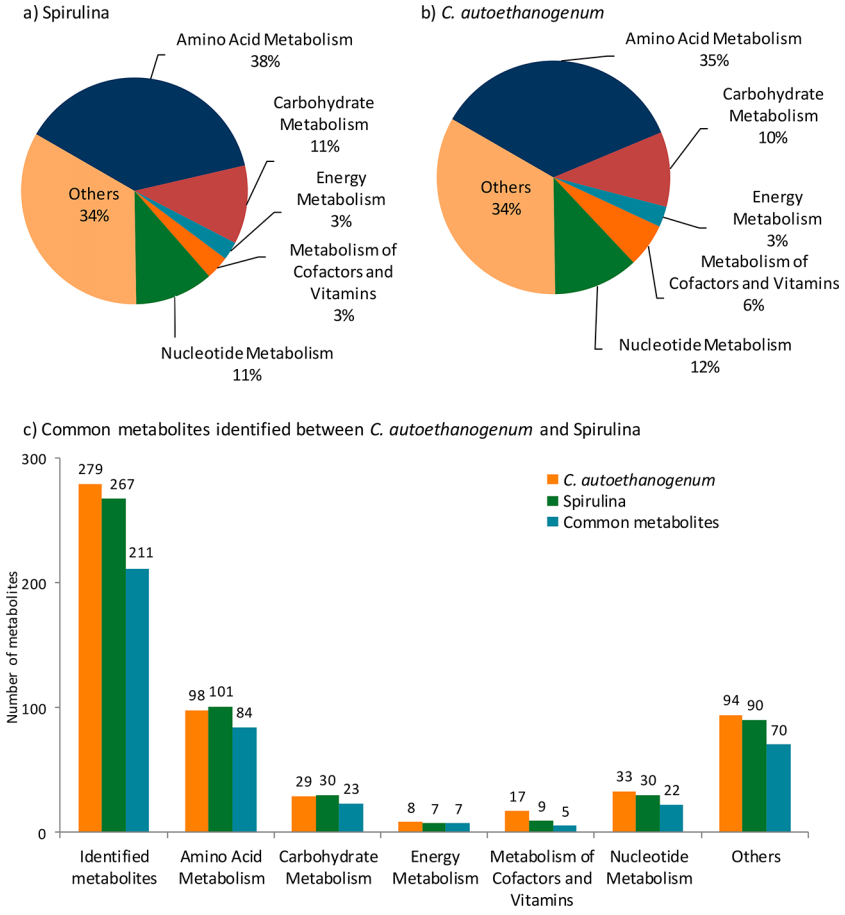


Figure 1. Comparison of the identified metabolite classes from (a) spirulina and (b) *C. autoethanogenum*. In total, 267 and 279 metabolites were tentatively identified using untargeted LC-MS profiling in the extracts of spirulina and *C. autoethanogenum*, respectively. (c) Metabolites common to the two species according to metabolite class. The two species were found to have 211 metabolites in common.

extracted bioreactor-batch samples were analyzed in a single analytical run with pooled QC samples interspaced every 10 samples. The concentrations of the metabolites of *C. autoethanogenum* in the samples were determined using standard calibration curves, and the obtained concentrations were normalized to the corresponding sample ODs of the bioreactor runs.

Strain and Culture Conditions of *C. autoethanogenum*. A *C. autoethanogenum* strain (DSM 10061) was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH culture collection (DSMZ, Braunschweig, Germany). The bacteria were revived by growth on a YTF agar medium, as stated elsewhere,[28] in an anaerobic cabinet (Don Whitley, West Yorkshire, U.K.) at 37 °C. The CO-gas-shift experiment was conducted using 3 L water-jacketed bioreactors in a continuous setup (BIOFLO 115, New Brunswick Scientific, Edison, NJ). In each bioreactor, a *C. autoethanogenum* seed culture was inoculated into a 1.5 L aqueous culture medium (LanzaTech, Skokie, IL).[29] Details of the continuous CO-fed bioreactor cultivation of *C. autoethanogenum*, the medium composition, and the bioreactor conditions are given in the Supporting Information.

Sample Preparation for *C. autoethanogenum* Intracellular Metabolite Determination. The bioreactor samples of *C. autoethanogenum* were harvested when the OD measurements at 600 nm (OD_{600}) reached 3.95. Three samples (1 mL each) of the cultures were collected every day throughout each experiment (35 days); the samples were immediately cooled down on ice to arrest metabolism and centrifuged at 10 000*g* for 5 min at 4 °C. The supernatants were then removed thoroughly, and the cell pellets were snap frozen in liquid nitrogen and kept at −80 °C for the LC-IDMS analysis.

Four different methods were evaluated for the extraction of the polar and semipolar intracellular metabolites of *C. autoethanogenum*: (1) solvent extraction (2:1:1 chloroform/methanol/water), (2) freeze−thaw extraction, (3) homogenization, and (4) sonication. For the first two methods, the cell pellets were resuspended in 150 μL of U-^{13}C-IS extract and vortexed for 30 s; then, 150 μL of water and 300 μL of chloroform were added, and the solutions were kept overnight at −20 °C (for the solvent extraction) or snap-frozen using liquid nitrogen (for the freeze−thaw method). Then, samples were centrifuged (10 000*g*, 5 min, 4 °C), and their aqueous phases were analyzed with LC-MS. For the homogenization method, 0.5 g of acid-washed glass beads (Sigma-Aldrich, Gillingham, U.K.) were used to disrupt the cells using Precellys homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France),[30] and the supernatants were analyzed with LC-MS. For the final method, sonication, the cell pellets were resuspended in 0.5 mL of deionized water, sonicated, centrifuged, and analyzed.

DOI: 10.1021/acs.analchem.7b04758
Anal. Chem. 2018, 90, 4470−4477

Table 1. Selection of a suitable method for extracting the metabolites of *C. autoethanogenum*

	extraction method (recovery %)					
				solvent extraction		
metabolite	freeze−thaw	homogenization	sonication	2:1:1[a]	1:2:2[b]	1:1[c]
fumarate	79 ± 9	57 ± 3	69 ± 0.39	99 ± 5	63 ± 8	77 ± 6
lactate			104 ± 0.02	101 ± 6	58 ± 11	69 ± 6
malate	51 ± 5	28 ± 2	98 ± 0.32	94 ± 4	70 ± 9	81 ± 9
citrate	43 ± 4	12 ± 7	88 ± 0.51	94 ± 4	80 ± 16	91 ± 5
glyoxylate	—	—	—	95 ± 19	—	—
2-ketoisovalerate	31 ± 4	35 ± 0.2	53 ± 0.11	—	60 ± 2	74 ± 2
pyruvate	12 ± 4	8 ± 3	16 ± 0.26	107 ± 6	59 ± 12	80 ± 2
succinate	—	—	126 ± 0.22	103 ± 6	63 ± 3	78 ± 3

[a]Chloroform/methanol/water. [b]Acetonitrile/methanol/water. [c]Methanol/water.

RESULTS AND DISCUSSION

Optimization of the LC-HRMS Method for the Simultaneous Quantification of *C. autoethanogenum* Metabolites. The optimized ZIC-*p*HILIC LC-HRMS method provided the quantitative analysis of a standard mixture of 74 metabolites of different classes and structurally related analogues simultaneously within a single 15 min analytical run compared with a similar 45 min method reported by others.[31,32] In this optimized method, organic acids were eluted at 4−9 min, amino acids were eluted at 8−12.5 min, and other energy molecules and intermediates were eluted between 7 and 9.5 min. The method provided improved separation of organic acids; isobaric compounds, such as dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate; and phosphorylated sugars (glucose 6-phosphate and fructose 6-phosphate), making the method suitable for their quantification without the need for the longer HILIC LC-MS or MS/MS analyses employed by others (see Supporting Information Figure S-1).[15,33,34] The main advantages of the developed method are that it provides (1) rapid separation and confirmed identification of many key intracellular metabolites in 15 min, (2) improved annotation of metabolite peaks compared with other LC-MS methods, and (3) the convenience of using commercially available ^{13}C spirulina over biosynthesized ^{13}C IS.

Characterization and Extraction of Commercially Available Spirulina as a Potential Source of IS for LC-IDMS. ^{13}C-Labeled lyophilized cell powder, obtained commercially from spirulina grown on a ^{13}C carbon source, was investigated as a potential source of U-^{13}C-labeled metabolites for use as multiple U-^{13}C IS for quantitative determination of intracellular *C. autoethanogenum* metabolites using LC-IDMS. The intracellular compositions of spirulina and *C. autoethanogenum* were analyzed by LC-HRMS and compared to evaluate if there was sufficient overlap in metabolites. Figure 1 shows that a similar range and number of metabolites were tentatively identified in both species. The full set of identified metabolites in both species is listed in Supporting Information Table S-1. The observed classes of metabolites were mainly related to amino acid metabolism (38% in spirulina and 35% in *C. autoethanogenum*), carbohydrate metabolism (11% in spirulina and 10% in *C. autoethanogenum*), and nucleotide metabolism (11% in spirulina and 12% in *C. autoethanogenum*). A total of 211 identified metabolites were cross-detected between the two species; 187 (88.63%) of these metabolites were found ^{13}C-labeled in the extract of U-^{13}C spirulina. Many of these are key metabolites in amino acid, nucleotide, carbohydrate, TCA, and energy metabolism pathways in *C. autoethanogenum*, confirming that the spirulina extract is an acceptable source of U-^{13}C-labeled IS for the LC-IDMS method described here. Others have generated ^{13}C-IS mixtures by growing organisms such as *E. coli* or yeast on $^{13}C_6$-glucose,[35−37] but these approaches are more complex and expensive than the use of a commercial source of ^{13}C-labeled IS. We show that the number of identified metabolites and metabolite classes in spirulina are similar to those identified for *E. coli*[13,25,38] and more than those reported for *Saccharomyces cerevisiae*,[10,39] the most common species used to biosynthesize ^{13}C IS for bacterial LC-IDMS. Thus, the use of readily available ^{13}C spirulina is a potential alternative to these biogenerated approaches. Furthermore, the previous studies use the same organisms for producing ^{13}C-IS extracts for absolute quantification,[10,38,40] but the LC-IDMS method developed here can be easily transferred to various organisms that cannot be grown on ^{13}C-glucose as the sole carbon source.

The extraction of ^{13}C metabolites from spirulina was investigated using a range of concentrations of spirulina powder (0.05−0.5%) with methanol and 2:1:1 chloroform/methanol/water. Sufficient concentrations of the ^{13}C metabolites were extracted using 0.5% (or greater) spirulina powder in methanol (Supporting Information Figure S-2). The stability and the recovery of ^{13}C-labeled key metabolites from U-^{13}C spirulina were also examined. Extracts of cell pellets of *C. autoethanogenum* spiked with U-^{13}C spirulina were kept at 4 °C for 36 h and analyzed at different time intervals with the optimized LC-HRMS; 53 U-^{13}C metabolites were detected and adequately recovered (in the range of 80−120%), and 43 of those metabolites were found to be stable (≥80%) with suitable signal-to-noise ratios (≥10, i.e., ≥LOQ) for use as U-^{13}C IS (Supporting Information Figure S-3). Furthermore, their ^{13}C-isotopic purities in the pure extracts of U-^{13}C spirulina (n = 13) showed that there was minimal ^{12}C present, with the majority of the ^{13}C metabolites having isotopic purities greater than 95% (Supporting Information Figure S-4). This indicates a good coverage of about 60% of the *C. autoethanogenum* metabolites under investigation. The wide spectrum of metabolites identified in the spirulina powder in this study shows the potential of extending the use of labeled spirulina as a readily available source of ^{13}C IS for the absolute quantification of *C. autoethanogenum* metabolites and for potential applications using LC-IDMS.

Optimization of the Bacterial-Sample Preparation for the Extraction of Intracellular Metabolites. A set of eight key intracellular metabolites of *C. autoethanogenum* was used to optimize the extraction procedure. The extraction of the metabolites via homogenization and freeze−thawing gave <60

DOI: 10.1021/acs.analchem.7b04758
Anal. Chem. 2018, 90, 4470−4477

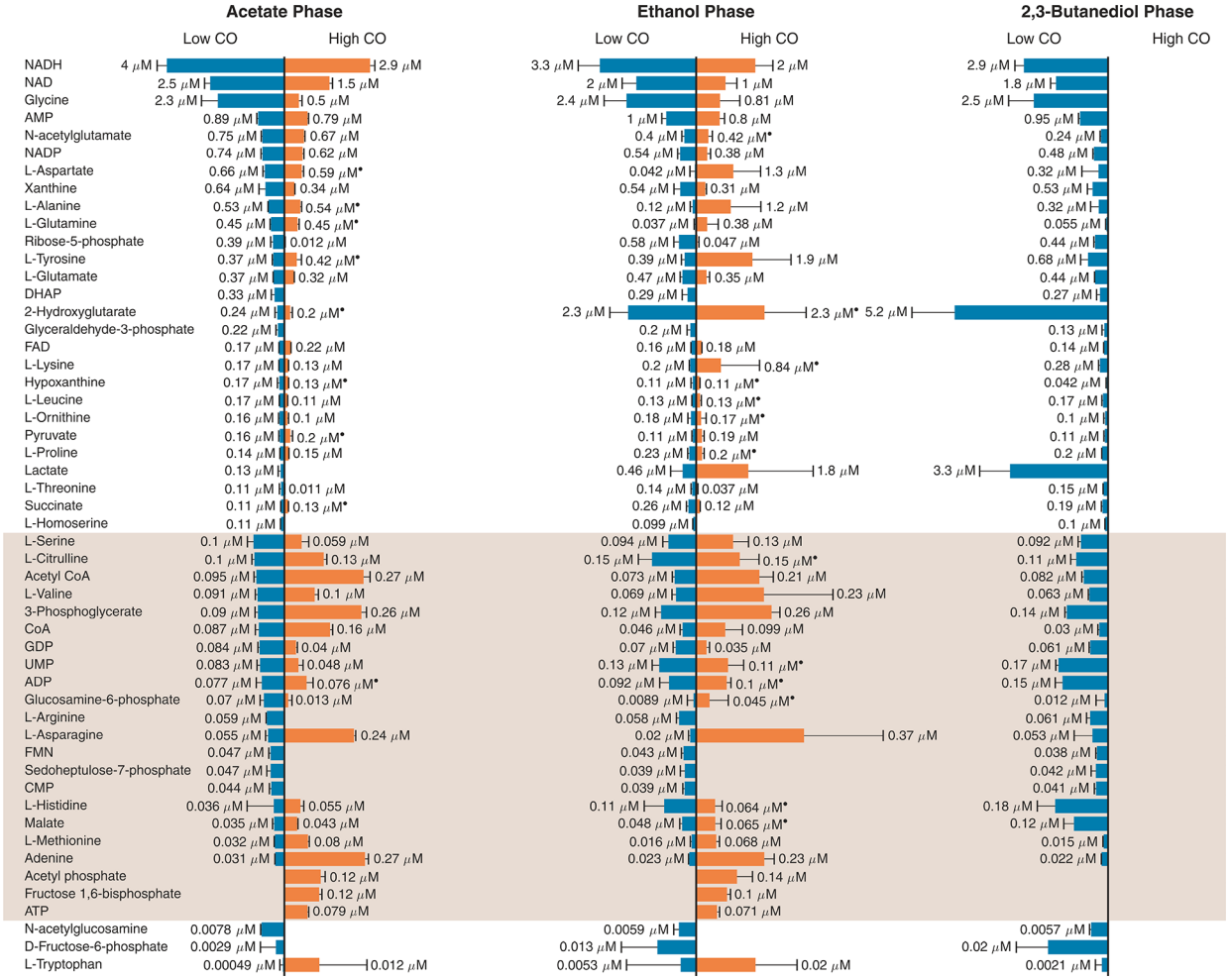


Figure 2. Measured levels of *C. autoethanogenum* metabolites in low- and high-CO-fed bioreactor samples quantified using the developed LC-IDMS method. The daily measured concentrations of metabolites (n = 3) were averaged at each steady state to provide a means of direct comparison between the two bioreactor batches fed with low CO (blue bars) and high CO (orange bars) for 35 days. Insignificant differences in the measured concentrations of the metabolites in the low- and the high-CO bioreactors (p-values ≤0.05) are indicated with dots (●), and the error bars (SD) estimate both the analytical and biological variability within each bioreactor steady state. Different scaling factors of metabolite concentrations have been employed to improve the visual comparisons between the steady states of the two bioreactors: the top (white) has a scale of 1, middle (gray) has a scale of 10, and the bottom (white) has a scale of 100.

and 80% recoveries, respectively, for all of the metabolites. Extraction using sonication gave an 88% recovery for all of the metabolites except for fumarate, 2-ketoisovalerate, and pyruvate, for which lower recoveries of 69, 53, and 16%, respectively, were observed. For the solvent extraction, the proportion of solvents used was first evaluated using different ranges of solvent combinations. The use of the chloroform/methanol/water (2:1:1) extraction gave satisfactory results, with 84% or higher recoveries for all of the metabolites (Table 1).

The use of cold-solvent extraction and sonication as extraction methods gave better recoveries compared with the use of homogenization and freeze−thawing, which was consistent with other studies.[41,42] However, lower recovery percentages for some of the metabolites were observed with sonication. This might reflect the increased oxidation processes of these metabolites, which is consistent with observations reported by Ohashi et al.[43] For the solvent extraction, Lee et al.[44] recommended the use of methanol alone for the extraction of intracellular metabolites of *C. acetobutylicum* for GC-MS, but this solvent was found to be less suitable in our study. This might be because most of the nonpolar metabolites were retained in the chloroform layer, giving less interference and cleaner chromatography of the polar metabolites in the aqueous phase. This is supported by the successful use of chloroform in varied proportions of chloroform/methanol/water, including 3:10:1,[45] 2.5:2.5:1,[43] and 2:2:1.[46] Hence, solvent extraction with 2:1:1 chloroform/methanol/water was selected for preparing the *C. autoethanogenum* samples for LC-IDMS.

Validation of LC-IDMS. The validation results of the LC-IDMS method are summarized in Supporting Information Table S-2. All of the responses of the metabolites were linear over the working range of the calibration curves with $R^2 \geq 0.99$. The precision and accuracy of the method at low (5 μM), medium (10 μM), and high (50 μM) concentrations were found to be within 15% RSD for 49 out of the 74 metabolites and within 20% for all of the metabolites. The LODs and LOQs of the pure standards were found to be within 1−500 nM and 1−1000 nM, respectively, for the majority of the metabolites (66 out of 74) except for 2-hydroxyglutarate, glucose 6-phosphate, glyceraldehyde-3-phosphate, lactate, L-serine, L-tyrosine, L-valine, and NADH, for which higher LODs (1−5

DOI: 10.1021/acs.analchem.7b04758
Anal. Chem. 2018, 90, 4470−4477

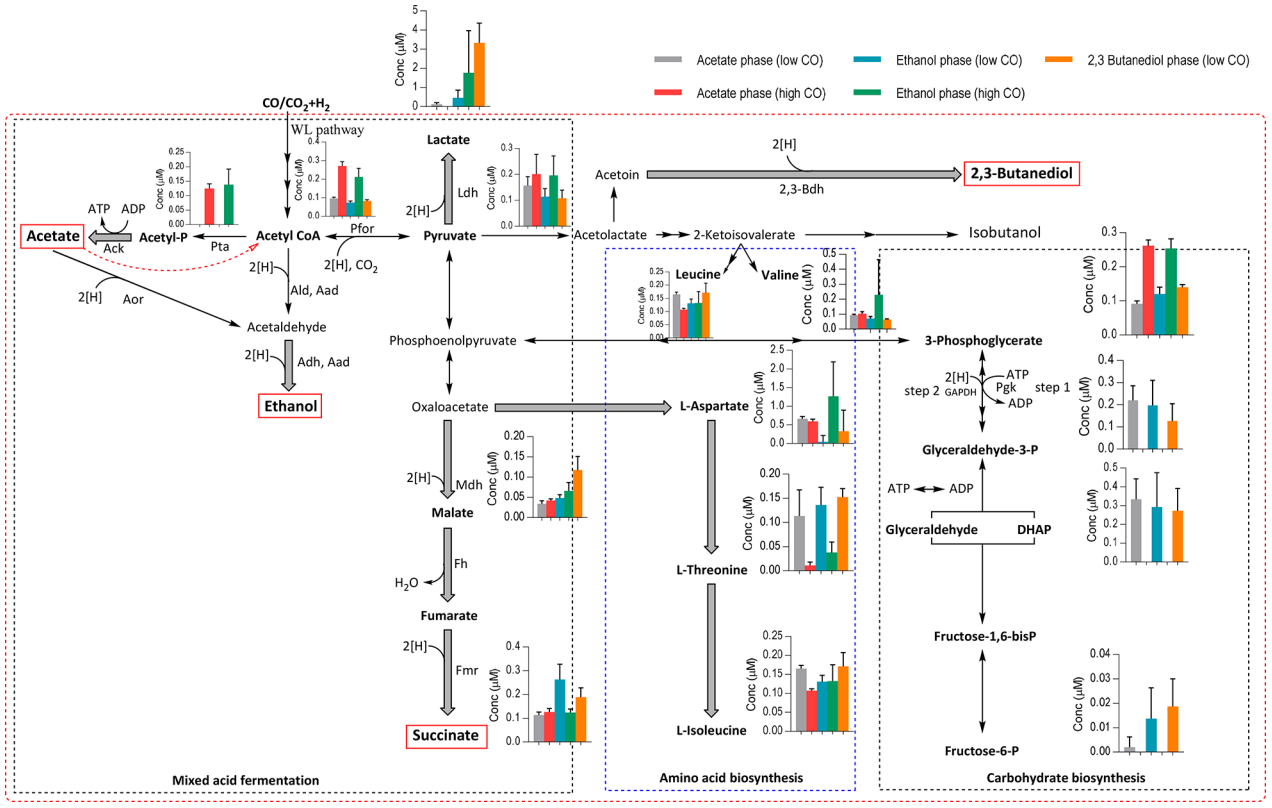


Figure 3. Different levels of intracellular metabolites mapped onto the main pathways of *C. autoethanogenum* involved in CO-gas fermentation. The bar graphs represent the levels of metabolites during acetate−low-CO (gray bars), acetate−high-CO (red bars), ethanol−low-CO (blue bars), ethanol−high-CO (green bars), and 2,3-butanediol−low-CO (orange bars) steady states.

μM) and LOQs (5−10 μM) were observed. The performance of the ZIC-*p*HILIC column was evaluated by monitoring the RTs of the selected metabolites using a QC sample (n = 11) interspaced within the analytical run of the extracted samples of *C. autoethanogenum* (24−36 h). Adequate reproducibility was observed in which the RTs of all of the metabolites were within the range of RSDs of 0.07−0.94% (see Supporting Information Table S-3). This result demonstrated that the multistep-gradient optimization with a flow-rate ramp from 300 to 400 μL/min not only improved the method throughput to 15 min but also shortened the longer equilibration time usually required with HILIC columns.[31,32]

The precision and accuracy of the quantitative measurements of nucleotides and organic acids were found to be improved compared with other similar ^{12}C-LC-MS-based methods.[47,48] The sensitivity achieved with the method is notably higher when compared with previous methods reported for nucleotides,[13,49,50] amino acids,[13,51] and organic acids[13,51] using similar methods. A full list of metabolites comparing the method's performance with those of similar reported studies is available in Supporting Information Table S-3. Our results indicate that the use of orbital-trap MS could achieve a comparable or even higher sensitivity than that of the triple-quadrupole-based detection for some metabolites without the need to employ MS/MS.

Profiling of Intracellular Metabolites of *C. autoethanogenum* to Optimize CO-Feed as a Sole Carbon Source. The developed LC-IDMS method was then applied to monitor the metabolic changes linked to the production of acetate, ethanol, and 2,3-butanediol between two bioreactor batches fed with CO as the sole carbon source at low- and high-flow rates (Supporting Information Figure S-5). LC-IDMS successfully quantified 52 metabolites in *C. autoethanogenum* in the daily samples analyzed over 35 days. A full list of metabolites and their measured concentrations over time are available in Supporting Information Table S-4. The measured levels of metabolites were averaged at each steady state to provide a means of direct comparison between the two bioreactors (Figure 2). In the high-CO-fed bioreactor, low levels of metabolites such as glycine, xanthine, ribose-5-phosphate, sedoheptulose-7-phosphate, NADH, and AMP were observed during both the acetogenic and solventogenic phases compared with those in the low-CO-fed bioreactor, whereas lactate, alanine, asparagine, tyrosine, acetyl CoA, acetyl phosphate, and 3-phosphoglycerate started to accumulate over time.

Mapping the selected set of metabolites to the main pathways of *C. autoethanogenum* enhances our understanding of the possible metabolic changes associated with high- and low-CO-fed conditions (Figure 3). For instance, the increased levels of acetyl CoA and acetyl phosphate in the high-CO-fed bioreactor may signal an impaired production of ethanol by aldehyde dehydrogenase (Ald), alcohol dehydrogenase (Adh), or aldehyde oxidoreductase (Aor). Recently, Marcellin et al. reported that Adh and Aor were up-regulated during CO fermentation in *C. autoethanogenum*.[52] It is believed that Aor and Adh play an important role in the production of ethanol from acetate[53,54] and acetyl CoA[52] in acetogens. This is consistent with the results from the low-CO-fed bioreactor, indicating that a high level of CO intake may down-regulate these enzymes and hence produce a lower level of ethanol.

High CO flow may affect gluconeogenesis in *C. autoethanogenum*. This is indicated by the absence of glyceradehyde-3-

DOI: 10.1021/acs.analchem.7b04758
Anal. Chem. 2018, 90, 4470−4477

phosphate, DHAP, and fructose-6-phosphate, whereas increased levels of 3-phosphoglycerate in all of the phases and lactate in the solventogenic phase were found. In *C. autoethanogenum*, pyruvate is converted to either lactate, phosphoenolpyruvate (the precursor in the TCA cycle and gluconeogenesis), or acetolactate.[55] Therefore, these results suggest that phosphoglycerate kinase and glyceraldehyde 3-phosphate dehydrogenase, enzymes in gluconeogenesis (Figure 3), are downregulated in high CO flows in favor of increased production of lactate, which is consistent with other studies.[52,56]

CONCLUSION

A new LC-IDMS method was successfully developed, validated for the quantification of bacterial metabolites, and applied to *C. autoethanogenum*. The method demonstrated the potential of using readily available labeled microorganisms as an easy route of obtaining multiple U-^{13}C IS. LC-IDMS, with the use of these multiple IS, provides a powerful approach for the quantification of a range of key intracellular metabolites. We have successfully analyzed more than 1300 samples with this method. Our approach offers improved quantitative pathway profiling for systematic investigations of metabolic alterations and is suitable for many applications requiring intra- and extracellular metabolite profiling of microorganisms.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.7b04758.

HPLC methods, culture conditions of *C. autoethanogenum*, continuous CO-fed bioreactor cultivation of *C. autoethanogenum*, extracted-ion chromatograms (XIC) of some key intracellular metabolites of *C. autoethanogenum* in comparison with the ^{13}C spirulina metabolites analyzed with the developed LC-IDMS method, extraction of the U-^{13}C-spirulina powder using different percentages of methanol or 2:1:1 chloroform/methanol/water, stabilities of the U-^{13}C spirulina metabolites spiked in extracts of *C. autoethanogenum*, quality of the selected U-^{13}C-labeled metabolites used as internal standards for LC-IDMS in the extract of U-^{13}C spirulina, production of ethanol and 2,3-butanediol by *C. autoethanogenum* (PDF)

Putatively identified metabolites in spirulina extracts, putatively identified metabolites in *C. autoethanogenum*, and the common metabolites between spirulina and *C. autoethanogenum*; validation of the developed LC-IDMS method showing the LODs, LOQs, precision, accuracy, and coverage of IS; LC-IDMS performance compared with those of other similar methods; concentrations of the metabolites in the low- and high-CO-fed bioreactors (XLSX)

AUTHOR INFORMATION

Corresponding Author

*E-mail: david.barrett@nottingham.ac.uk. Tel.: +44 (0) 115 951 5062. Fax: +44 (0) 115 951 5102.

ORCID

Salah Abdelrazig: 0000-0001-6231-1267

Present Address

[§]S.S.: Evonik Nutrition & Care GmbH, Kantstraße 2, 33790 Halle/Westphalia, Germany.

Author Contributions

[⊥]S.S. and S.A. contributed equally.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the Biotechnology and Biological Sciences Research Council [grant number BB/K00283X/1] (UK BBSRC) as part of the BBSRC Longer and Larger Grant GASCHEM; and the industrial partner LanzaTech Inc.

REFERENCES

(1) Sumner, L. W.; Mendes, P.; Dixon, R. A. *Phytochemistry* **2003**, *62*, 817−836.

(2) Lei, Z.; Huhman, D. V.; Sumner, L. W. *J. Biol. Chem.* **2011**, *286*, 25435−25442.

(3) Fairchild, J. N.; Horvath, K.; Gooding, J. R.; Campagna, S. R.; Guiochon, G. *J. Chromatogr. A* **2010**, *1217*, 8161−8166.

(4) Kol, S.; Merlo, M. E.; Scheltema, R. A.; de Vries, M.; Vonk, R. J.; Kikkert, N. A.; Dijkhuizen, L.; Breitling, R.; Takano, E. *Appl. Environ. Microbiol.* **2010**, *76*, 2574−2581.

(5) Luo, B.; Groenke, K.; Takors, R.; Wandrey, C.; Oldiges, M. *J. Chromatogr. A* **2007**, *1147*, 153−164.

(6) Lu, W.; Clasquin, M. F.; Melamud, E.; Amador-Noguez, D.; Caudy, A. A.; Rabinowitz, J. D. *Anal. Chem.* **2010**, *82*, 3212−3221.

(7) Koc, H.; Mar, M.-H.; Ranasinghe, A.; Swenberg, J. A.; Zeisel, S. H. *Anal. Chem.* **2002**, *74*, 4734−4740.

(8) Meija, J.; Mester, Z. *Anal. Chim. Acta* **2008**, *607*, 115−125.

(9) Sargent, M.; Harte, R.; Harrington, C. *Guidelines for Achieving High Accuracy in Isotope Dilution Mass Spectrometry (IDMS)*; RSC: Cambridge, 2002.

(10) Wu, L.; Mashego, M. R.; van Dam, J. C.; Proell, A. M.; Vinke, J. L.; Ras, C.; van Winden, W. A.; van Gulik, W. M.; Heijnen, J. J. *Anal. Biochem.* **2005**, *336*, 164−171.

(11) Cui, J.; Zhang, J.; Zhu, X.; Bai, F.; Feng, Y.; Guan, W.; Cui, Q. *Anal. Lett.* **2013**, *46*, 2767−2786.

(12) Sandberg, T. E.; Long, C. P.; Gonzalez, J. E.; Feist, A. M.; Antoniewicz, M. R.; Palsson, B. O. *PLoS One* **2016**, *11*, e0151130.

(13) Bajad, S. U.; Lu, W.; Kimball, E. H.; Yuan, J.; Peterson, C.; Rabinowitz, J. D. *J. Chromatogr. A* **2006**, *1125*, 76−88.

(14) Xu, Y.-F.; Lu, W.; Rabinowitz, J. D. *Anal. Chem.* **2015**, *87*, 2273−2281.

(15) Su, X.; Lu, W.; Rabinowitz, J. D. *Anal. Chem.* **2017**, *89*, 5940−5948.

(16) Köpke, M.; Mihalcea, C.; Liew, F.; Tizard, J. H.; Ali, M. S.; Conolly, J. J.; Al-Sinawi, B.; Simpson, S. D. *Appl. Environ. Microbiol.* **2011**, *77*, 5467−5475.

(17) Abrini, J.; Naveau, H.; Nyns, E.-J. *Arch. Microbiol.* **1994**, *161*, 345−351.

(18) Köpke, M.; Gerth, M. L.; Maddock, D. J.; Mueller, A. P.; Liew, F.; Simpson, S. D.; Patrick, W. M. *Appl. Environ. Microbiol.* **2014**, *80*, 3394.

(19) Rauniyar, N.; McClatchy, D. B.; Yates, J. R., III *Methods* **2013**, *61*, 260−268.

(20) Mayers, M. D.; Moon, C.; Stupp, G. S.; Su, A. I.; Wolan, D. W. *J. Proteome Res.* **2017**, *16*, 1014.

(21) Savas, J. N.; Park, S. K.; Yates, J. R. Proteomic Analysis of Protein Turnover by Metabolic Whole Rodent Pulse-Chase Isotopic Labeling and Shotgun Mass Spectrometry Analysis. In *Quantitative Proteomics by Mass Spectrometry*, Sechi, S., Ed.; Springer: New York, 2016; pp 293−304.

(22) Tautenhahn, R.; Böttcher, C.; Neumann, S. *BMC Bioinf.* **2008**, *9*, 504.

DOI: 10.1021/acs.analchem.7b04758
Anal. Chem. 2018, 90, 4470−4477

(23) Scheltema, R. A.; Jankevics, A.; Jansen, R. C.; Swertz, M. A.; Breitling, R. *Anal. Chem.* **2011**, *83*, 2786−2793.
(24) Creek, D. J.; Jankevics, A.; Burgess, K. E. V.; Breitling, R.; Barrett, M. P. *Bioinformatics* **2012**, *28*, 1048−1049.
(25) Kim, D.-H.; Achcar, F.; Breitling, R.; Burgess, K. E.; Barrett, M. P. *Metabolomics* **2015**, *11*, 1721−1732.
(26) Sumner, L.; Amberg, A.; Barrett, D.; Beale, M.; Beger, R.; Daykin, C.; Fan, T. M.; Fiehn, O.; Goodacre, R.; Griffin, J.; Hankemeier, T.; Hardy, N.; Harnly, J.; Higashi, R.; Kopka, J.; Lane, A.; Lindon, J.; Marriott, P.; Nicholls, A.; Reily, M.; Thaden, J.; Viant, M. *Metabolomics* **2007**, *3*, 211−221.
(27) *Guidance for Industry: Bioanalytical Method Validation*; Food and Drug Administration, Center for Drug Evaluation and Research: Silver Spring, MD, 2013.
(28) Humphreys, C. M.; McLean, S.; Schatschneider, S.; Millat, T.; Henstra, A. M.; Annan, F. J.; Breitkopf, R.; Pander, B.; Piatek, P.; Rowe, P.; Wichlacz, A. T.; Woods, C.; Norman, R.; Blom, J.; Goesman, A.; Hodgman, C.; Barrett, D.; Thomas, N. R.; Winzer, K.; Minton, N. P. *BMC Genomics* **2015**, *16*, 1085.
(29) Mihalcea, C.; Haryanto, P. A fermentation method. WO2013147621A1, March 28, 2013.
(30) Bertin Technologies, Precellys, The Science of Lysing, 2013. http://www.ozyme.fr/gammes/ber/pdf/ber_precellys_livret.pdf
(31) Zhang, R.; Watson, D. G.; Wang, L.; Westrop, G. D.; Coombs, G. H.; Zhang, T. *J. Chromatogr. A* **2014**, *1362*, 168−179.
(32) Creek, D. J.; Jankevics, A.; Breitling, R.; Watson, D. G.; Barrett, M. P.; Burgess, K. E. V. *Anal. Chem.* **2011**, *83*, 8703−8710.
(33) Danaceau, J. P.; Chambers, E. E.; Fountain, K. J. *Bioanalysis* **2012**, *4*, 783−794.
(34) Havlíková, L.; Vlčková, H.; Solich, P.; Nováková, L. *Bioanalysis* **2013**, *5*, 2345−2357.
(35) Lu, W.; Su, X.; Klein, M. S.; Lewis, I. A.; Fiehn, O.; Rabinowitz, J. D. *Annu. Rev. Biochem.* **2017**, *86*, 277−304.
(36) Bennett, B. D.; Kimball, E. H.; Gao, M.; Osterhout, R.; Van Dien, S. J.; Rabinowitz, J. D. *Nat. Chem. Biol.* **2009**, *5*, 593.
(37) Park, J. O.; Rubin, S. A.; Xu, Y.-F.; Amador-Noguez, D.; Fan, J.; Shlomi, T.; Rabinowitz, J. D. *Nat. Chem. Biol.* **2016**, *12*, 482.
(38) Bennett, B. D.; Yuan, J.; Kimball, E. H.; Rabinowitz, J. D. *Nat. Protoc.* **2008**, *3*, 1299−1311.
(39) Ibáñez, A. J.; Fagerer, S. R.; Schmidt, A. M.; Urban, P. L.; Jefimovs, K.; Geiger, P.; Dechant, R.; Heinemann, M.; Zenobi, R. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 8790−8794.
(40) Mashego, M. R.; Wu, L.; Van Dam, J. C.; Ras, C.; Vinke, J. L.; Van Winden, W. A.; Van Gulik, W. M.; Heijnen, J. J. *Biotechnol. Bioeng.* **2004**, *85*, 620−628.
(41) Tian, J.; Sang, P.; Gao, P.; Fu, R.; Yang, D.; Zhang, L.; Zhou, J.; Wu, S.; Lu, X.; Li, Y.; Xu, G. *J. Sep. Sci.* **2009**, *32*, 2281−2288.
(42) Booth, S. C.; Weljie, A. M.; Turner, R. J. *Front. Microbiol.* **2015**, *6*, 827.
(43) Ohashi, Y.; Hirayama, A.; Ishikawa, T.; Nakamura, S.; Shimizu, K.; Ueno, Y.; Tomita, M.; Soga, T. *Mol. BioSyst.* **2008**, *4*, 135−147.
(44) Lee, S.-H.; Kim, S.; Kwon, M.-A.; Jung, Y. H.; Shin, Y.-A.; Kim, K. H. *Biotechnol. Bioeng.* **2014**, *111*, 2528−2536.
(45) Krall, L.; Huege, J.; Catchpole, G.; Steinhauser, D.; Willmitzer, L. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2009**, *877*, 2952−2960.
(46) Drazic, A.; Kutzner, E.; Winter, J.; Eisenreich, W. *PLoS One* **2015**, *10*, e0125823.
(47) Pabst, M.; Grass, J.; Fischl, R.; Léonard, R.; Jin, C.; Hinterkörner, G.; Borth, N.; Altmann, F. *Anal. Chem.* **2010**, *82*, 9782−9788.
(48) Ibáñez, A. B.; Bauer, S. *Biotechnol. Biofuels* **2014**, *7*, 145.
(49) Qian, T.; Cai, Z.; Yang, M. S. *Anal. Biochem.* **2004**, *325*, 77−84.
(50) Klawitter, J.; Schmitz, V.; Klawitter, J.; Leibfritz, D.; Christians, U. *Anal. Biochem.* **2007**, *365*, 230−239.
(51) Lu, W.; Kimball, E.; Rabinowitz, J. D. *J. Am. Soc. Mass Spectrom.* **2006**, *17*, 37−50.
(52) Marcellin, E.; Behrendorff, J. B.; Nagaraju, S.; DeTissera, S.; Segovia, S.; Palfreyman, R. W.; Daniell, J.; Licona-Cassani, C.; Quek, L.-e.; Speight, R.; Hodson, M. P.; Simpson, S. D.; Mitchell, W. P.; Kopke, M.; Nielsen, L. K. *Green Chem.* **2016**, *18*, 3020−3028.
(53) Mock, J.; Zheng, Y.; Mueller, A. P.; Ly, S.; Tran, L.; Segovia, S.; Nagaraju, S.; Köpke, M.; Dürre, P.; Thauer, R. K. *J. Bacteriol.* **2015**, *197*, 2965−2980.
(54) Köpke, M.; Held, C.; Hujer, S.; Liesegang, H.; Wiezer, A.; Wollherr, A.; Ehrenreich, A.; Liebl, W.; Gottschalk, G.; Dürre, P. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 13087−13092.
(55) Brown, S. D.; Nagaraju, S.; Utturkar, S.; De Tissera, S.; Segovia, S.; Mitchell, W.; Land, M. L.; Dassanayake, A.; Köpke, M. *Biotechnol. Biofuels* **2014**, *7*, 40.
(56) Liew, F.; Henstra, A. M.; Winzer, K.; Köpke, M.; Simpson, S. D.; Minton, N. P. *mBio* **2016**, *7*, e00427-16.

DOI: 10.1021/acs.analchem.7b04758
Anal. Chem. 2018, 90, 4470−4477