High-Throughput Quantitative Metabolomics: Workflow for Cultivation, Quenching, and Analysis of Yeast in a Multiwell Format

Jennifer Christina Ewald, Stephanie Heux, Nicola Zamboni

High-Throughput Quantitative Metabolomics: Workflow for Cultivation, Quenching, and Analysis of Yeast in a Multiwell Format

Jennifer Christina Ewald,†,‡ Stéphanie Heux,† and Nicola Zamboni*,†

Institute of Molecular Systems Biology, and Life Science Zurich Ph.D. Program on Systems Biology of Complex Diseases, ETH Zurich, Wolfgang-Pauli Strasse 16, 8093 Zurich, Switzerland

Metabolomics is a founding pillar of quantitative biology and a valuable tool for studying metabolism and its regulation. Here we present a workflow for metabolomics in microplate format which affords high-throughput and yet quantitative monitoring of primary metabolism in microorganisms and in particular yeast. First, the most critical step of rapid sampling was adapted to a multiplex format by using fritted 96-well plates for cultivation, which ensure fast sample transfer and permit us to use well-established quenching in cold solvents. Second, extensive optimization of large-volume injection on a GC/TOF instrument provided the sensitivity necessary for robust quantification of 30 primary metabolites in 0.6 mg of yeast biomass. The metabolome profiles of baker's yeast cultivated in fritted well plates or in shake flasks were equivalent. Standard deviations of measured metabolites were between 10% and 50% within one plate. As a proof of principle we compared the metabolome of wild-type Saccharomyces cerevisiae and the single-deletion mutant Δsdh1, which were clearly distinguishable by a 10-fold increase of the intracellular succinate concentration in the mutant. The described workflow allows the production of large amounts of metabolome samples within a day, is compatible with virtually all liquid extraction protocols, and paves the road to quantitative screens.

The importance of metabolomics in the context of quantitative biology is steadily growing.[1-4] Metabolite concentrations provide a direct snapshot of the state of metabolism, which reflect the integrated output of a multitude of complex interactions, and therefore can be used, for example, for diagnostics or to unravel circuits of metabolic regulation. The increased demand is further accompanied by the proliferation of novel in silico approaches to gain deeper insight from metabolomics data.[5-10] Further the high-resolution, broad-spectrum, and relatively inexpensive nature of metabolomics promote it to become a large-scale screening tool for investigating traits related to metabolism.[4,11] To exploit this potential, high-throughput and multiplexed protocols and methods are required to generate and analyze large metabolomics data sets.

For microorganisms, several examples exist to demonstrate how metabolome data can elucidate the effects of environmental and genetic perturbations on primary metabolism.[12-16] Although physiological characterization of both bacterial and yeast cells can be routinely performed in 96-well format,[17-19] metabolomics experiments are typically carried out in shake flasks or bioreactors.[13,15,20,21] These offer both a well-controlled environment as well as sufficient amounts of biomass to produce samples but are time-intensive and allows only limited throughput.

Although the potential applications of metabolomics experiments done in multiwell plate format are very attractive, the transfer to miniaturized cultures is hindered by two issues. First, rapid inactivation of metabolism (quenching) is technically difficult

* To whom correspondence should be addressed. E-mail: zamboni@imsb.biol.ethz.ch. Phone: +41 44 633 31 41. Fax: +41 44 633 10 51.

† Institute of Molecular Systems Biology.

‡ Life Science Zurich Ph.D. Program on Systems Biology of Complex Diseases.

(1) Wang, Q. Z.; Wu, C. Y.; Chen, T.; Chen, X.; Zhao, X. M. *Appl. Microbiol. Biotechnol.* **2006**, *70*, 151–161.

(2) Griffin, J. L. *Philos. Trans. R. Soc. London* **2006**, *361*, 147–161.

(3) Nielsen, J.; Oliver, S. *Trends Biotechnol.* **2005**, *23*, 544–546.

(4) Mapelli, V.; Olsson, L.; Nielsen, J. *Trends Biotechnol.* **2008**, *26*, 490–497.

(5) Ku¨mmel, A.; Panke, S.; Heinemann, M. *Mol. Syst. Biol.* **2006**, *2*, 0034.

(6) Weckwerth, W.; Morgenthal, K. *Drug Discovery Today* **2005**, *10*, 1551–1558.

(7) Cakir, T.; Patil, K. R.; Onsan, Z.; Ulgen, K. O.; Kirdar, B.; Nielsen, J. *Mol. Syst. Biol.* **2006**, *2*, 50.

(8) Hageman, J. A.; Hendriks, M. M.; Westerhuis, J. A.; van der Werf, M. J.; Berger, R.; Smilde, A. K. *PLoS ONE* **2008**, *3*, e3259.

(9) Nikerel, I. E.; van Winden, W. A.; Verheijen, P. J.; Heijnen, J. J. *Metab. Eng.* **2009**, *11*, 20–30.

(10) Mendes, P.; Camacho, D.; de la Fuente, A. *Biochem. Soc. Trans.* **2005**, *33*, 1427–1429.

(11) Hollywood, K.; Brison, D. R.; Goodacre, R. *Proteomics* **2006**, *6*, 4716–4723.

(12) Raamsdonk, L. M.; Teusink, B.; Broadhurst, D.; Zhang, N.; Hayes, A.; Walsh, M. C.; Berden, J. A.; Brindle, K. M.; Kell, D. B.; Rowland, J. J.; Westerhoff, H. V.; van Dam, K.; Oliver, S. G. *Nat. Biotechnol.* **2001**, *19*, 45–50.

(13) Mashego, M. R.; Jansen, M. L. A.; Vinke, J. L.; van Gulik, W. M.; Heijnen, J. J. *FEMS Yeast Res.* **2005**, *5*, 419–430.

(14) Phelps, T. J.; Palumbo, A. V.; Beliaev, A. S. *Curr. Opin. Biotechnol.* **2002**, *13*, 20–24.

(15) Ishii, N.; Nakahigashi, K.; Baba, T.; Robert, M.; Soga, T.; Kanai, A.; Hirasawa, T.; Naba, M.; Hirai, K.; Hoque, A.; Ho, P. Y.; Kakazu, Y.; Sugawara, K.; Igarashi, S.; Harada, S.; Masuda, T.; Sugiyama, N.; Togashi, T.; Hasegawa, M.; Takai, Y.; Yugi, K.; Arakawa, K.; Iwata, N.; Toya, Y.; Nakayama, Y.; Nishioka, T.; Shimizu, K.; Mori, H.; Tomita, M. *Science (Washington, DC, U.S.)* **2007**, *316*, 593–597.

(16) Ohashi, Y.; Hirayama, A.; Ishikawa, T.; Nakamura, S.; Shimizu, K.; Ueno, Y.; Tomita, M.; Soga, T. *Mol. BioSyst.* **2008**, *4*, 135–147.

(17) Duetz, W. A.; Ruedi, L.; Hermann, R.; O'Connor, K.; Buchs, J.; Witholt, B. *Appl. Environ. Microbiol.* **2000**, *66*, 2641–2646.

(18) Fischer, E.; Sauer, U. *Nat. Genet.* **2005**, *37*, 636–640.

(19) Blank, L. M.; Ku¨pfer, L.; Sauer, U. *Genome Biol.* **2005**, *6*, R49.

(20) Wittmann, C.; Hans, M.; van Winden, W. A.; Ras, C.; Heijnen, J. J. *Biotechnol. Bioeng.* **2005**, *89*, 839–847.

(21) Wu, L.; van Dam, J.; Schipper, D.; Kresnowati, M.; Proell, A. M.; Ras, C.; van Winden, W. A.; van Gulik, W. M.; Heijnen, J. J. *Appl. Environ. Microbiol.* **2006**, *72*, 3566–3577.

to achieve with parallel cultures. In quantitative metabolomics with either mass spectrometry (MS) or nuclear magnetic resonance detection is done off-line after metabolites have been extracted from the cells. To obtain a snapshot of metabolism that reflects the in vivo state, it is a prerequisite to quench metabolism sufficiently fast and efficiently to avoid that during sampling or extraction, residual enzymatic activity or degradation bias the results. Since the turnover for some intermediates or cofactors is on the subsecond time scale, the general rule of thumb prescribes to complete quenching in 1 s. With a single sample from shake flasks or bioreactors, quenching is typically achieved by spraying the cell suspension into solvents precooled at very low temperatures.[22-24] Organic solvents are used to avoid the formation of ice crystals well below 0 °C. The widely used protocol for yeast uses methanol/water mixture at -40 °C.[23] This allows immediate quenching of metabolism and subsequent removal of medium by centrifugation. Methanol minimally affects membrane integrity if the exposure time does not exceed few minutes.[24,25] Unfortunately, bacteria are less tolerant to organic solvents and metabolite leakage is frequently observed.[26] Nevertheless, liquid extraction remains the preferred method used both directly[22,27] or after quenching.[28] When attempting to transfer these protocols to microplate format, sample handling, suspension, and heat transfer become problematic.

Second, detection on generally used gas chromatography MS (GC/MS) or liquid chromatography MS(LC/MS) instruments or NMR often require that several milligrams of dry biomass are sampled to quantify most key or abundant metabolites.[22,28,29] When sampling from a microwell format during midexponential phase, however, less than 1 mg of dry biomass is available for analysis. The decrease in sample amount has to be compensated by an increase in sensitivity. Notably, washing of cell pellets from medium compounds after quenching is always incomplete, and large amounts of extracellular contaminants (e.g., sugars, buffers, salts, fermentation products) are still present in the cell extracts. This further complicates the accurate quantification of low-abundant intracellular intermediates in a sometimes overwhelming matrix.

Here, we present a comprehensive metabolomics workflow for parallel cultivation, quenching, extraction, and robust quantification of primary metabolites by gas chromatography time-of-flight (GC/TOF) MS. To validate the workflow we use baker's yeast (*Saccharomyces cerevisiae*) which is a commonly used eukaryotic model organism and was among the first organisms to be studied by metabolomics approaches. Cultivation, quenching, and extraction techniques have been previously investigated and optimized.[23,25,30,31] Here, we rely on these established protocols and adapt them to microplate format introducing novel 96-well fritted plates for multiplexed sample cultivation and handling.

To quantify the metabolome in microplate extracts, we opted to utilize GC/TOF because it is a widely used platform for both targeted and profiling studies.[32-34] GC/TOF is suitable for detection of all volatile compounds as well as small polar compounds after derivatization.[35-37] We focus our attention on the intermediates of central carbon metabolism and therefore utilize two commonly used silylations protocols thatsafter methoxymationsuse either trimethylsilyl (TMS) or *tert*-butyldimethylsilyl (TBDMS) agents.[36] To cope with the low biomass availability, an overall increase in GC/TOF sensitivity is attained here by large-volume injection with a temperature-programmable injector. Large-volume injection has been extensively used for analysis of highly volatile compounds and solvents, for example, in pesticide analysis.[38,39] However, removing solvents and silylation reagents commonly used for metabolomics without losing analytes during solvent venting is a challenge due to their high vaporization temperatures, and parameters have to be optimized independently for the two derivatization procedures.

EXPERIMENTAL SECTION

Strains and Cultivation. For all experiments we used *S. cerevisiae* CEN.PK 113-7D (haploid, mat R) (Euroscarf, Germany). The prototroph sdh1 knockout mutant YKL148c::kanMX4 was constructed in the same background by Blank et al.[19] Yeast cells were cultivated in 600 μL of glucose minimal medium (Verduyn et al.[40]), supplemented with 10 mM potassium phthalate buffer (pH 5), in 96-well deep well plates with fritted bottoms (Nunc, U.S.A., no. 278011). A 4 mm glass bead was added to each well to improve mixing. Plates were sealed with a gas-permeable lid and incubated at 30 °C, shaking at 300 rpm with a radius of 5 cm. Comparative studies in shake flasks were performed with identical medium in 500 mL flasks (30 °C, 250 rpm, 30 mL total filling volume). Cells growth was monitored by the optical density (600 nm) in a SpectraMax Plus plate reader (Molecular Devices, U.S.A.). For metabolomics experiments, the optical density was determined at three time points: approximately 4 h, 2 h, and 5 min before sampling, thereby removing a total of 150 μL culture volume.

Quenching and Extraction. *Microwell Plates.* The quenching and extraction procedure applied was adapted from de Konig and

(22) Brauer, M. J.; Yuan, J.; Bennett, B. D.; Lu, W.; Kimball, E.; Botstein, D.; Rabinowitz, J. D. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 19302–19307.
(23) de Koning, W.; van Dam, K. *Anal. Biochem.* **1992**, *204*, 118–123.
(24) Villas-Boas, S. G.; Hojer-Pedersen, J.; Akesson, M.; Smedsgaard, J.; Nielsen, J. *Yeast* **2005**, *22*, 1155–1169.
(25) Canelas, A.; Ras, C.; ten Pierick, A.; van Dam, J.; Heijnen, J.; van Gulik, W. *Metabolomics* **2008**, *4*, 226–239.
(26) Bolten, C. J.; Kiefer, P.; Letisse, F.; Portais, J.-C.; Wittmann, C. *Anal. Chem.* **2007**, *79*, 3843–3849.
(27) Schaub, J.; Schiesling, C.; Reuss, M.; Dauner, M. *Biotechnol. Prog.* **2006**, *22*, 1434–1442.
(28) Koek, M. M.; Muilwijk, B.; van der Werf, M. J.; Hankemeier, T. *Anal. Chem.* **2006**, *78*, 1272–1281.
(29) 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.
(30) Castrillo, J. I.; Hayes, A.; Mohammed, S.; Gaskell, S. J.; Oliver, S. G. *Phytochemistry* **2003**, *62*, 929–937.
(31) Gonzalez, B.; Francois, J.; Renaud, M. *Yeast* **1997**, *13*, 1347–1355.
(32) MacKenzie, D. A.; Defernez, M.; Dunn, W. B.; Brown, M.; Fuller, L. J.; Herrera, S. R. M. S. d.; Gu¨nther, A.; James, S. A.; Eagles, J.; Philo, M.; Goodacre, R.; Roberts, I. N. *Yeast* **2008**, *25*, 501–512.
(33) Humston, E. M.; Dombek, K. M.; Hoggard, J. C.; Young, E. T.; Synovec, R. E. *Anal. Chem.* **2008**, *80*, 8002–8011.
(34) Kopka, J. *J. Biotechnol.* **2006**, *124*, 312–322.
(35) Dunn, W. B.; Ellis, D. I. *Trends Anal. Chem.* **2005**, *24*, 285–294.
(36) Halket, J. M.; Waterman, D.; Przyborowska, A. M.; Patel, R. K.; Fraser, P. D.; Bramley, P. M. *J. Exp. Bot.* **2005**, *56*, 219–243.
(37) Katona, Z. F.; Sass, P.; Molna´r-Perl, I. *J. Chromatogr., A* **1999**, *847*, 91– 102.
(38) Mol, H. G. J.; Althuizen, M.; Janssen, H.-G.; Cramers, C. A.; Brinkman, U. A. T. *HRC & CC, J. High Resolut. Chromatogr. Chromatogr. Commun.* **1996**, *19*, 69–79.
(39) Stan, H.-J.; Linkerha¨gner, M. *J. Chromatogr., A* **1996**, *727*, 275–289.
(40) Verduyn, C.; Postma, E.; Scheffers, W. A.; Van Dijken, J. P. *Yeast* **1992**, *8*, 501–517.

van Dam and Gonzalez et al.[23,31] Each well of a 48 square deep well plate (Interchim, France) was filled with 3.6 mL of 60% methanol, 10 mM ammonium acetate (pH 7.5) and precooled to −40 °C. This plate was placed in a vacuum manifold with the cultivation plate on top. Cells and medium were transferred from the cultivation plate to the quenching plate by applying vacuum. In this step, two wells (450 μL each) of the cultivation plate were pooled to one well in the quenching plate. Pooling was necessary because the wells of commercially available fritted plates can only accommodate half of the volume of deep well plates. The quenching plate with cells in the quenching solution was immediately placed into a dry ice−ethanol bath (−50 °C) for 2 min. Cells were pelleted by centrifugation (4000 rpm, 5 min, −9 °C), and the supernatant removed by inversion of the plate. The plate was returned to the cold bath. Cells were resuspended in precooled 1 mL of 75% ethanol, 10 mM ammonium acetate (pH 7.5). An amount of 100 μL of internal standard mix was added, containing 0.5 nmol glutaric acid and 0.5 nmol norvaline solved in the same ethanol/buffer mixture. Extraction was completed by heating to 80 °C in a water bath for 3 min, vortexing every 30 s. Cell debris were removed by centrifugation (4000 rpm, 10 min, −9 °C). The extract was transferred to a fresh plate and stored at −80 °C (up to 4 weeks) until analysis.

Shake Flasks. The 0.9 mL samples from shake flasks were quenched in 3.6 mL of 60% methanol, 10 mM ammonium acetate (pH 7.5) in 15 mL reaction tubes, cooled to −40 °C. Cells were pelleted by centrifugation (5000 rpm, 5 min, −9 °C), and the supernatant was discarded. Pellets were immediately frozen in liquid nitrogen. For extraction, 1 mL of 75% ethanol, 10 mM ammonium acetate, preheated to 80 °C was added. An amount of 100 μL of the same internal standard mix as described above was added. Samples were incubated in a water bath at 80 °C for 3 min, vortexing every minute. Cell debris were removed by centrifugation, and the extract was transferred to fresh tubes.

Analytics. *GC/TOF Measurement.* Extracts were transferred to 1.7 mL plastic microtubes and completely dried in a vacuum centrifuge. In a first derivatization step, 20 μL of methoxyamine solution (20 mg/mL methoxyamine hydrochloride (Supleco) in pyridine (analytical grade, Merck)) were added and samples were incubated in a thermomixer for 90 min at 40 °C and 1000 rpm. Then 7.5 μL aliquots were transferred to GC glass vials and sealed with magnetic caps. The second derivatization step was performed automatically and just-in-time using an MPS2 sampler controlled by Maestro software (Gerstel, Germany). For TMS derivatization 15 μL of *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (Fluka) were added to each vial, and the mixture was incubated for 30 min at 40 °C, shaking at 250 rpm. For TBDMS derivatization 15 μL of *N*-*tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide with 1% *tert*-butyl-dimethylchlorosilane (Fluka) were added, and the mix- ture was incubated 60 min at 80 °C, shaking at 250 rpm. An amount of 5 μL of TMS derivatives or 2 μL of TBDMS derivatives was applied to a 30 m GC column (HP-5-MS, 30 m × 0.25 mm × 0.25 μm, Agilent) using a CIS4-injector (Gerstel, Germany) and cold large-volume injection as detailed in Table 1. Separation was achieved by applying a GC temperature gradient from 60 to 300 °C (Table 1) with a constant helium carrier flow of 1.3 mL/min. To prevent carryover a 5 min wash run with 1 μL of pyridine was included between each sample. The detector was a time-of-flight mass spectrometer (Pegasus III, Leco, U.S.A.), scanning from m/z 40 to 800 with an acquisition rate of 40 spectra/s at unit resolution. Peak detection and assignment were performed with ChromaTOF software 2.52 (Leco, U.S.A.). Peaks were identified with a spectral library recorded by the authors by injecting pure compounds (Sigma, highest available quality). Calibration curves were spiked with ^{13}C-labeled yeast biomass extract (equivalent to approximately 0.2 mg cell dry weight) to induce the matrix effects typical for yeast extracts.[41] Amino acids were normalized to the internal standard norvaline (5 nmol per sample); all other analytes were normalized to glutaric acid (5 nmol per sample). Internal standards were added to samples during extraction and to calibration standards before drying.

Table 1. Optimized Injection and Gas Chromatography Parameters for TMS- and TBDMS-Derivatized Samples

	TMS	TBDMS
	Injector	
volume injected	5 μL	2 μL
injection speed	0.3 μL/s	0.3 μL/s
solvent venting (He)	100 mL/min	100 mL/min
venting time	15 s	8 s
initial temp	40 °C	40 °C
hold	0.5 min	0.5 min
heating rate	12 °C/min	12 °C/min
final temp	300 °C	300 °C
	GC Parameters	
initial temp	60 °C	60 °C
hold	0.5 min	0.5 min
rate 1	25 °C/min	30 °C/min
hold	0.5 min	0.5 min
rate 2	20 °C/min	20 °C/min
final temp	300 °C	300 °C
final hold	1.5 min	2.5 min

High-Performance Liquid Chromatography. Extracellular metabolites were determined as described previously[42] with an Agilent HP1100 high-performance liquid chromatography (HPLC) system (Agilent, U.S.A.), equipped with an Aminex HPX-87H column (BioRad, U.S.A.) heated to 60 °C. The mobile phase was 5 mM H_2SO_4 (isocratic). The compounds were detected and quantified with a refractive index and an UV−vis detector.

Adenylate Energy Charge. The adenylate energy charge[43] (AEC) is a measure for the energy state of a cell and is defined as

$$\mathrm{AEC} = ([\mathrm{ATP}] + 0.5[\mathrm{ADP}])/([\mathrm{ATP}] + [\mathrm{ADP}] + [\mathrm{AMP}])$$

Adenylatephosphate concentrations were determined by ion-pairing reversed-phase liquid chromatography[44] on a 4000 QTrap LC/MS/MS instrument (MDS Sciex/Applied Biosystems, U.S.A.). Peak detection and integration were performed using Analyst 1.4.2. software.

RESULTS AND DISCUSSION

Optimization of GC/TOF Analysis. Parallel cultivation for high-throughput purposes is bound to downscaling of culture

(41) Bu¨scher, J. M.; Czernik, D.; Ewald, J. C.; Sauer, U.; Zamboni, N. *Anal. Chem.* **2009**, *81*, 2135–2143.
(42) Heer, D.; Sauer, U. *Microb. Biotechnol.* **2008**, *1*, 497–506.
(43) Atkinson, D. E. *Biochemistry* **1968**, *7*, 4030–4034.
(44) Luo, B.; Groenke, K.; Takors, R.; Wandrey, C.; Oldiges, M. *J. Chromatogr., A* **2007**, *1147*, 153–164.

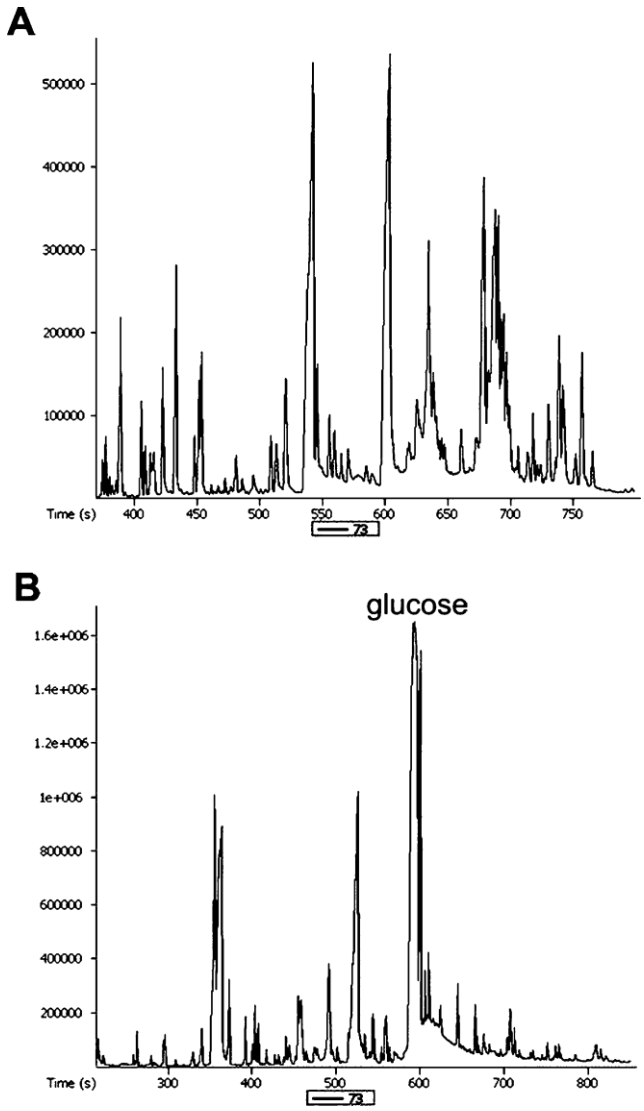

Figure 1. Representative chromatogram obtained for TBDMS (A) and TMS (B) derivatization with 0.6 mg of dry biomass. The extracted ion trace *m/z*) 73 shows all silanized compounds.

volumes and therefore imposes additional challenges in sensitivity. Although most protocols for intracellular metabolomics often require several to hundreds of milligrams of cell dry weight,[22,28,29] microplate cultures yield less than 1 mg of cell mass per well. To compensate for the reduced sample amount, we utilized a temperature-programmable injection program and solvent venting to increase the injection volume beyond the limits imposed by splitless injection. However, because of the presence of highly abundant metabolites (e.g., glutamate) or medium contaminants (e.g., glucose, phosphate, phthalate) in intracellular extracts it is not possible to increase at will the injection volume without overloading column or detector. To ensure throughput, we set out to seek for the best tradeoff to quantify both low and highly abundant species with a single injection and acquisition run. Hence, we empirically optimized the parameters for large-volume injection based on a set of 56 primary metabolites including amino and organic acids, sugars, sugar phosphates, and purines (Supporting Information Table 1). These metabolites cover most of central carbon metabolism and represent important pathway such as glycolysis, TCA cycle, and amino acid synthesis.

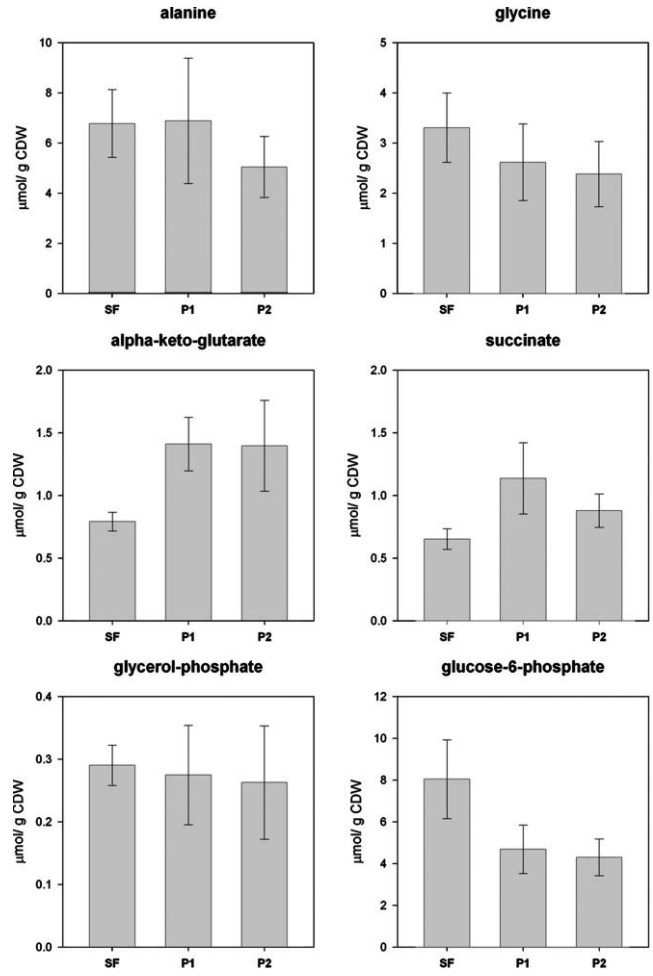

Figure 2. Representative metabolite concentrations of *S. cerevisiae* grown in shake flasks and fritted plates. Abbreviation: SF, shake flasks (*n*) 4); P1, fritted plate no. 1 (*n*) 20 wells); P2, fritted plate no. 2 (*n*) 20 wells). The complete data set is reported in Supporting Information Table 3.

Because of their polar nature, these analytes are not volatile in the native form but require chemical derivatization before gas chromatography. We used the well-established methoxymation and silylation with either TMS or TBDMS groups (Figure 1). TMS groups are relatively small and allow thorough derivatization of molecules with numerous hydroxylic and carboxylic groups or amines. Hence, polyols such as sugars or sugar phosphates are visible in a GC/MS spectrum after TMS derivatization. A downside of TMS is that primary amines can be become doubly derivatized. Since the second derivatization is slow, it typically results in multiple chromatographic peaks that complicate quantification when the derivatization procedure is not carefully controlled.[45] In contrast, TBDMS groups are more bulky: although sugars fail to become fully derivatized and thus volatile, amines are rapidly modified with single TBDMS groups. In comparison to TMS, TBDMS is more selective for organic and amino acids and better suited for their quantification. The chromatograms of TBDMS-derivatized extracts contain less background from sugars (Figure 1).

Empirical optimization based on yeast extracts eventually led to the methods described in Table 1. The peculiarities of TMS and TBDMS derivatives and matrixes resulted in diverging

(45) Kanani, H. H.; Klapa, M. I. *Metab. Eng.* **2007**, *9*, 39–51.

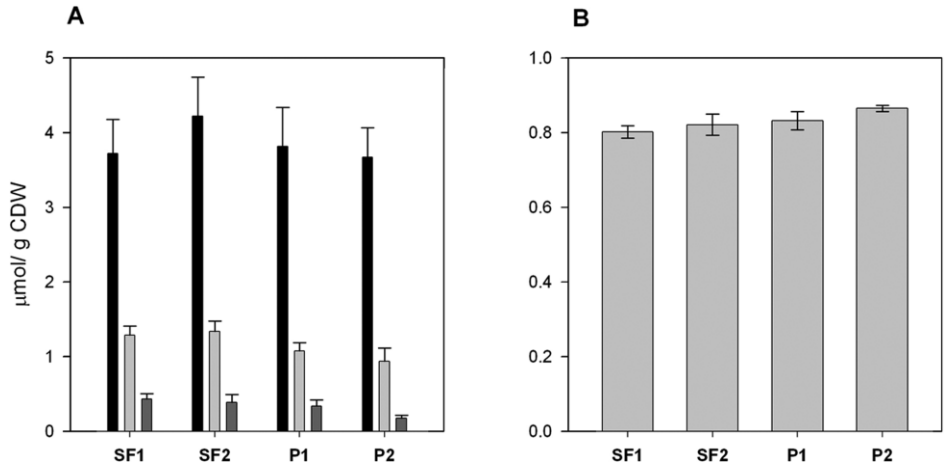

Figure 3. Nucleotide amounts in *S. cerevisiae* grown in shake flasks and fritted plates determined from six aliquots or six wells, respectively. (A) Absolute concentrations of ATP (black), ADP (light gray), and AMP (dark gray). (B) Adenylate energy charge. Abbreviations: SF1, shake flask 1; SF2, shake flask 2; P1, fritted plate 1; P2, fritted plate 2.

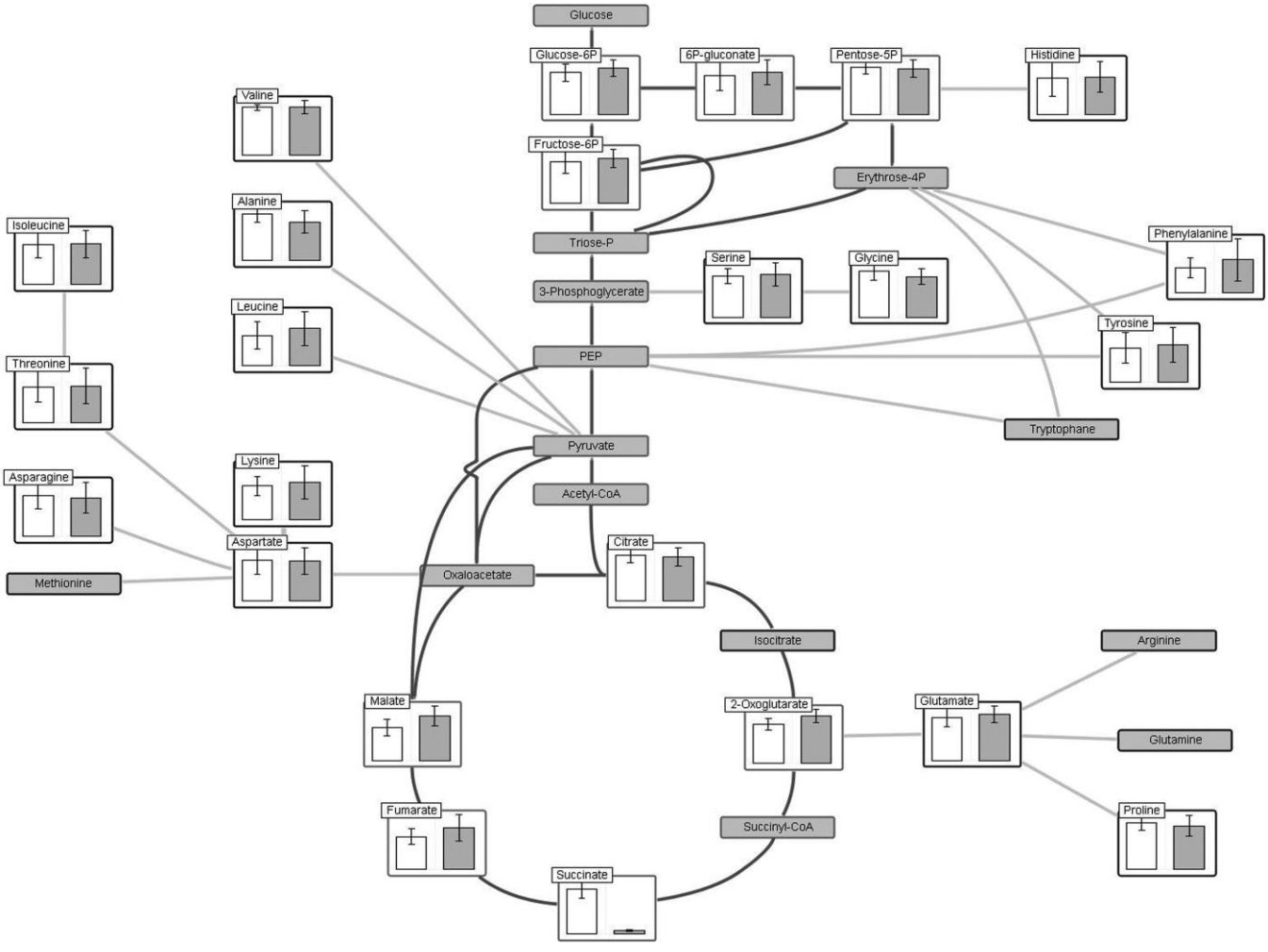

Figure 4. Comparison of metabolite concentrations in the sdh1 deletion mutant (white bars, n) 10) and the parent *S. cerevisiae* strain (gray bars, n) 10) during batch growth in fritted 96-well plates.

injection parameters. This enabled to robust detection in extracts of the majority of compounds in our reference set. Given the hundreds of deconvoluted but unidentified peaks observed in a typical chromatogram, the spectrum of compounds targeted could be greatly increased if necessary (e.g., fatty acids).

Quantification. In biological matrix, we observed a linear response over 2-3 orders of magnitude ($R^2 > 0.98$, data not shown) for 40 of the 56 compounds tested. We were able to detect and quantify 30 metabolites from 900 μL of biomass at an optical density (600 nm) of 1 (0.6 mg cell dry weight), which

is the amount obtained from microplate cultivation in the presented protocol (Supporting Information Table 1). The limit of quantification was between 0.05 and 0.2 nmol per sample (4-16 pmol on column). This corresponds to cellular concentrations of approximately 0.065-0.26 μmol/g cell dry weight.

Quantification of compounds in biological samples is susceptible to errors due to matrix effects caused by highly abundant metabolites (e.g., glutamate) as well as media contaminants (for example, glucose, phosphates, or salts)[35,41] We observed that in the presented method matrix primarily modified derivatization efficiency and thus, to mimic the matrix effects typical for biological samples, all calibrations curves for quantification were determined by a dilution series of pure standards spiked with constant amounts of fully ^{13}C-labeled biomass extract (equivalent to 0.2 mg of biomass). To quantify the amount of metabolites in unknown extracts, it was then sufficient to use two nonla- beled internal standards for normalization: norvaline for amino acids and glutaric acid for all other compounds. These two compounds were selected in an empirical screen with additional candidates. Both compounds do not naturally occur in microbial samples (they could not be detected in up to 2 mg of biomass of yeast or *B. subtilis* samples), give clear mass traces for quantification, and are not as cost-intensive as labeled compounds. We also tested utilization of ^{13}C-labeled standards for some target metabolites. However, this strategy did not deliver significant improvements when compared with norvaline/ glutaric acid. In contrast, ^{13}C internal standards complicated automatic spectral identification and reduced the linear range due to column overloading.

Validation of the New Cultivation and Quenching Procedure. *Physiological Parameters.* To implement state-of-the-art quenching and extraction protocols in a multiwell format we cultivated yeast in 96-well fritted plates. A hydrophobic frit at the bottom of each well enables leak-free cultivation. At will, vacuum can be used to instantaneously (approximately 1 s) transfer cells and media into a second plate placed at the bottom and containing quenching solution. Batch growth of microbes in a 96 deep well plate format has already been thoroughly validated by Duetz et al.[17] Specifically, they showed that mass transfer of substrates and oxygen were not limiting microbial growth. To ensure that this holds also for the adapted setup using fritted plates, the growth rates of wild-type *S. cerevisiae* on glucose minimal medium was determined in shake flasks, standard micro deep well plates (1.2 mL volume), and the fritted cultivation plates (0.6 mL volume) by monitoring optical density over time. Growth rates in all three setups as well as biomass and byproduct yields as determined by HPLC analysis of the media were similar (Supporting Information Table 2). Oxygen proved not limiting as *S. cerevisiae* was able to grow normally in fritted well plates also on gluconeogenic substrates such as ethanol, which require a higher oxygen uptake (data not shown).

Physical Parameters. To assess potential retention of biomass in the vacuum-transfer step, we grew *S. cerevisiae* to midexponential phase and determined the optical density. The cells were vacuum-transferred to a fresh plate, and the optical density was remeasured. The recovery was >98% for all wells, and thus, we considered the loss in this step to be negligible. Further, it is critical that cells remain at low temperature during the quenching process to inhibit residual enzymatic activity. We therefore checked the temperature in the quenched cell solution immediately after quenching (-38 °C in central wells) and after centrifugation (-22 °C in outer wells). As cells remained cooled well below 0 °C at all times until extraction, we are confident that metabolic activity was sufficiently suppressed.

Metabolome. Next, we wanted to asses the reproducibility of the new metabolomics workflow. All 96 wells of two fritted plate were inoculated with an overnight preculture of wild-type yeast. After four doubling times (optical density of 1), metabolism was quenched by vacuum-transferring cells into cold methanol. Metabolites were extracted and quantified with GC/TOF as described above. Standard deviations within one plate ranged from 10% (e.g., valine) up to 50% (e.g., threonine). The average standard deviation was 30%, which is slightly larger than we typically observed in shake flasks. This can be expected due to the higher risks of imprecise handling when working in a multiplex format. We compared the obtained data to the metabolome of *S. cerevisiae* cultivated in four shake flasks, inoculated from the same preculture. Twenty-one of the tested compounds were identical or very similar in concentration between the two cultivation conditions, nine compounds showed a difference of more than 30%, thereof four compounds more than 50%. This might be due to the marginally higher growth and glucose uptake rates or small differences in the microenvironment in the different cultivation setups. This does not affect comparative studies within one setup. Some representative examples of metabolite concentrations of different compound classes are shown in Figure 2; a detailed summary can be found in Supporting Information Table 3.

Efficiency of Quenching. The AEC reflects the energy state of a cell and is a sensitive measure for the quality of quenching and sampling handling. Incomplete quenching or sample degradation result in lower ATP and thus AEC levels. Since ATP, ADP, and AMP are not measurable by GC instruments their concentrations were measured on an LC tandem mass spectrometry (MS/MS) system (Figure 3A). Both in shake flasks and in fritted well plates we found an AEC larger than 0.8 (Figure 3B), which matches the expected values for growing cells.[46] Another indicator for quenching efficiency of glucose grown cells is the ratio of glucose- 6-phosphate to fructose-6-phosphate. This ratio quickly drops if glucose influx stops before metabolism is arrested or samples warm up before extraction. Despite the differences in absolute concentrations observed here in fritted plates, the ratio of glucose-6-phosphate and fructose-6-phosphate is identical in fritted plates and shake flask grown cells (approximately 4.2). We conclude that the adapted quenching procedure is efficient and the sample processing described in this protocol is equally effective as established protocols.

Applying Microplate Metabolomics for Comparative Mutant Studies. In a proof-of-principle biological application we tested the effect of the deletion of the sdh1 gene, which encodes a subunit of the succinate dehydrogenase, a mitochondrial TCA cycle enzyme. *S. cerevisiae* wild-type and Δsdh1 mutants were grown and quenched in one microplate. The mutant strain showed a mild growth defect of 10%. Ten randomly selected extracts each were analyzed. We observed a clear difference in the concentration of intracellular succinate, which was 10-fold increased in the

(46) Ball, W. J., Jr.; Atkinson, D. E. *J. Bacteriol.* **1975**, *121*, 975–982.

mutant (Figure 4). All other concentrations, including those of other TCA intermediates, were indistinguishable or similar between the strains (Figure 4, Supporting Information Table 4). This shows that, although flux into the TCA cycle is very low on glucose-access conditions,[47] the substrate of the deleted reaction accumulates. Presumably because of the highly exothermic (i.e., irreversible) upstream reaction this accumulation does not propagate backward. This experiment demonstrates that metabolomics can be a useful high-throughput tool to identify, localize, or characterize mutations in yeast.

CONCLUDING REMARKS

We present for the first time a complete quantitative metabolomics workflow in a microplate format. The protocol allows the generation of 48 metabolome samples in parallel and can thus be a useful tool for high-throughput exploratory studies as well as screening for biotechnological applications. At this stage, increasing throughput to a 96-well format is only prevented by the physical dimensions of commercially available plates. Though the workflow presented here was designed for and validated with *S. cerevisiae*, the principle can be adapted to virtually any unicellular organism that grows in suspension or other quenching/extraction solvents.[24,25] The obtained data is highly reproducible and is in good agreement with data obtained from established protocols. An average standard deviation of 30% allows the detection of even small fold changes in the metabolome. The single-deletion mutant sdh1 is a good example that a genetic perturbation can lead to a localized, well-defined response in metabolism.

With this platform in place, analysis time becomes the limiting factor. The methods used here support a throughput of 50 samples per day and instrument. This strongly depends on the analytes of interest and can be increased by sacrificing chromatographic resolution and using rapid gradients.

In this study, we successfully quantified 30 metabolites in extracts from a set of 56 compounds, which were initially selected because of our interest in primary metabolism. This provides a good coverage of central carbon and amino acid metabolism. Nevertheless, 300–500 deconvoluted peaks are typically detected in a single GC/TOF run with TMS- or TBDMS-derivatized extracts. The amount of positively identified and quantifiable analytes is likely to increase with more comprehensive spectral libraries that include additional compound classes or intermediates of peripheral biosynthetic pathways. Complementing GC-based methods with LC-based methods could further broaden the spectrum of quantifiable metabolites, for example, to nucleotides or coenzyme A derivatives.[41] Similarly, sensitivity can be further increased by selective MS^2 detection at the cost of a nontargeted approach.

ACKNOWLEDGMENT

The authors thank Uwe Sauer for his support and helpful discussions. S.H. gratefully acknowledges funding by Marie Curie Intra-European Fellowships (F6P-2005).

SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

(47) Blank, L. M.; Sauer, U. *Microbiology* **2004**, *150*, 1085–1093.