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ORIGINAL ARTICLE

Fast Swinnex filtration (FSF): a fast and robust sampling and extraction method suitable for metabolomics analysis of cultures grown in complex media

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Abstract Liquid chromatography tandem mass spectrometry (LC–MS/MS) provides a powerful means to analyze intracellular metabolism. A prerequisite to accurate metabolomics analysis using LC–MS/MS is a robust sampling and extraction protocol. One unaddressed area in sampling is a detailed examination of a suitable method for anaerobic cultures grown in complex media. Given that a vast majority of bacteria are facultative or obligate anaerobes that grow to low biomass density and need to be cultured in complex media, a suitable sampling and extraction strategy for anaerobic cultures is needed. In this work, we develop a fast-filtration method using pressure-driven Swinnex® filters. We show that the method is fast enough to provide an accurate snapshot of intracellular metabolism, reduces matrix interference from the media to improve the number of compounds that can be detected, and is applicable to anaerobic and aerobic liquid cultures grown in a variety of culturing systems. Furthermore, we apply the fast filtration method to investigate differences in the absolute intracellular metabolite levels of anaerobic cultures grown in minimal and complex media.

Keywords LC–MS/MS · Intracellular metabolome · Anaerobic and complex liquid media cultures · Fast sampling and extraction

1 Introduction

Metabolomics has played an instrumental role in furthering our understanding of intracellular metabolism (Bennett et al. 2009; Nakahigashi et al. 2009; Jozefczuk et al. 2010; Doucette et al. 2011; Buescher et al. 2012; Xu et al. 2012a, b; Ibanez et al. 2013; Link et al. 2013; Taymaz-Nikerel et al. 2013). Liquid chromatography tandem mass spectrometry (LC–MS/MS) based methods provide a powerful approach to interrogate the metabolome by combining throughput and sensitivity (van Dam et al. 2002; Bajad et al. 2006; Cai et al. 2009; Buescher et al. 2010; Lu et al. 2010; Bennette et al. 2011). A prerequisite to accurate metabolomics analysis using LC–MS/MS is the optimization of the sampling and extraction protocol (Kimball and Rabinowitz 2006; Bolten et al. 2007). For intracellular metabolites with a turnover on the order of seconds or less, it must be fast enough to provide an accurate snapshot of metabolism, but also produce a suitable sample for analysis. For liquid cultures, meeting these demands is non-trivial, difficult to achieve, but critically important if meaningful data is to be generated.

Many bacteria are facultative or obligate anaerobes. Consequently, the ability to assay bacteria from anaerobic cultures to improve our understanding of their biochemistry for scientific, therapeutic, and industrial endeavors is highly relevant. Many of these bacteria require supplementation

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with complex nutrients such as yeast extract (YE), peptones, and blood components, among others, in order to be cultured in the lab. This presents unique challenges to LC–MS/MS-based metabolomics methods. Anaerobic cultures often reach a much lower biomass than aerobic cultures. For instance in our experience, stationary phase cultures of wild-type *E. coli* grown anaerobically in 4 g L^{-1} of M9 minimal media reach a culture density of approximately 0.25 gDW L^{-1} while stationary phase cultures of wild-type *E. coli* grown aerobically in the same media reach a culture density of approximately 1.35 gDW L^{-1}. As the biomass of the culture decreases, the interference from media components increases. This is particularly problematic for anaerobic cultures where the amount of organic acids found in the culture medium that are produced by fermentation hamper the ability to accurately measure intracellular organic acid levels. The problem of media interference is exacerbated for cultures grown with supplementation. A common supplement for auxotrophic strains of *E. coli* is YE, which encompasses the water-soluble portion of autolyzed yeast. Often the amount of YE added to the growth medium can be an order of magnitude greater than the culture density itself. This makes accurate differentiation of intracellular from extracellular components inherently problematic if they are not fully removed.

Many strategies exist for removing and differentiating intracellular from extracellular components. These include fast filtration (Bolten et al. 2007; Jozefczuk et al. 2010; Van Gulik et al. 2012), fast centrifugation (Buescher et al. 2010), and direct extraction either from liquid cultures, such as shake flasks (McCloskey et al. 2013) or pH controlled bioreactors (Taymaz-Nikerel et al. 2009, 2011, 2013; De Mey et al. 2010), or from cultures grown on filters (Rabinowitz and Kimball 2007; Bennett et al. 2008). Previous studies have shown that the time required to perform fast filtration with a typical filtration setup and vacuum pump is sufficient for compounds that turnover less quickly, but is not sufficient for the physiologically important compounds that turnover in the time frame of seconds (Bolten et al. 2007). Fast centrifugation appears to quench metabolism in a timely manner, but its application when working in an anaerobic chamber or with anaerobic cultures does not appear viable. Direct extraction provides the fastest means to quench metabolism. Unfortunately, the organic solvents needed to quench metabolism cause the bacterial membrane to become permeable, resulting in cell leakage and inaccurate measurement of the intracellular metabolome (Bolten et al. 2007; Canelas et al. 2008; Link et al. 2008). If the culture density is sufficient, such as in a pH controlled bioreactor, the sample can be directly extracted and dilutions can be employed in order to reduce matrix interference (Taymaz-Nikerel et al. 2009; Van Gulik et al. 2012). By taking a measurement of the culture filtrate in parallel, the intracellular concentrations can be determined from the difference of the whole broth and filtrate (Taymaz-Nikerel et al. 2009; Van Gulik et al. 2012). When the culture density is not sufficient to allow for dilutions, the direct extraction and application of the differential method can still be employed, but the number of compounds that can be analyzed accurately can be limited due to matrix interference (McCloskey et al. 2013). As an alternative, samples can be grown directly on the filter used to extract the culture (Kimball and Rabinowitz 2006; Bennett et al. 2008). However, this approach does not allow for multiple filtrate and/or broth samples to be taken from the same culture at different time-points or phases of growth. It also does not appear suitable for use with an anaerobic chamber (a popular culturing method) due to contamination of the chamber atmosphere with organic solvents if the extraction is performed in the chamber itself, or exposure to oxygen if the filter cultures are removed from the chamber prior to extraction.

Several automated devices have been constructed to assist in rapidly sampling liquid cultures from bioreactors (Schaefer et al. 1999; Lange et al. 2001; Mashego et al. 2003; Schaub et al. 2006; De Mey et al. 2010) as well as from flasks (Hiller et al. 2007; McCloskey et al. 2013). While improving the reliability of rapid sampling, the devices are optimized for specific culture conditions, which limit their broad use. For researchers who culture cells under a wide array of culturing systems, a more flexible sampling system is needed. This is particularly true if samples need to be obtained from cultures grown in an anaerobic environment or environmental samples need to be obtained from the field. Most devices are designed to be used in conjunction with a direct extraction of the liquid culture, which causes problems for low biomass cultures and cultures grown with supplementation. These problems include decreased column life-time, increased instrument maintenance, and reduced number of compounds that can be accurately quantified due to ion-suppression and media interference. Thus, an alternative sampling method that reduces the amount of media included with the cell biomass while still quenching metabolism fast enough (i.e., on an equivalent time-frame to that of direct extraction methods) to provide an accurate snap-shot of intracellular metabolite levels is needed.

In this work, we sought to develop a rapid sampling and extraction method that (1) can be applied to a wide range of liquid culturing systems and environments (including anaerobic environments), (2) that provides sufficient sampling and quenching speed to arrest cellular metabolism in order to provide an accurate snap-shop of the intracellular metabolome, and (3) that minimizes matrix-induced interference for accurate analysis by LC–MS/MS. We describe the steps taken to optimize a fast-filtration sampling and

extraction method implemented using pressure-driven Swinnex® filters (FSF) to meet these goals and the resulting optimized method. We show that by working with a syringe filter, we are able to quench metabolism at a comparable rate to that of the direct extraction approach. Further, we show that the method is applicable to sampling liquid cultures from a variety of culturing vessels and conditions, and allows for greater coverage of metabolites to be accurately quantified using LC–MS/MS. In addition, we apply the method to investigate differences in the absolute intracellular metabolite levels of anaerobic cultures grown in minimal media and media supplemented with YE.

2 Materials and methods

2.1 Chemicals and reagents

Water, methanol, acetonitrile, acetonitrile +0.1 % formic acid, and water used for extraction were purchased from Honeywell Burdick & Jackson® (Muskegon, MI). Ammonium formate and triethylammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO). YE was purchased from Fisher® Scientific (Pittsburgh, PA). Metabolically labeled internal standards were generated as described previously (McCloskey et al. 2013) from batch cultures of *E. coli* grown on uniformly labeled ^{13}C glucose, started from over-night pre-cultures of *E. coli* also grown on uniformly labeled ^{13}C glucose. Swinnex® filter holders and 0.45 µM filters (PES, mixed cellulose ester, and PVDF) were purchased from Millipore® (Billerica, MA).

2.2 Biological material and culture conditions

E. coli K12 MG1655 (ATCC 700926), obtained from the American Type Culture Collection (Manassas, VA), were grown in 4 g/L glucose M9 minimal media (Sambrook 2001) with trace elements (Fong et al. 2005) with or without 1 g/L of YE. Growth under aerobic batch consisted of shake flasks in a water bath maintained at 37C and aerated at 500 RPM. Growth in an aerobic pH controlled bioreactors was used in both batch mode and in glucose limited continuous culture mode at two different dilutions rates (0.31 and 0.44 h^{-1}) (see supplemental methods for more details of the chemostat experiments). The steady-state for glucose limited continuous cultures was achieved after 3–5 residence times and was verified by biomass measurements. Growth under anaerobic conditions consisted of shake flasks in an anaerobic chamber (COY; 37 °C; 10 % CO_2, balance N_2). Cultures were sampled during steady-state growth at an OD600 of 0.6 (aerobic batch), an OD600 of 1.0 (aerobic batch bioreactor), an OD600 of 3.0 (aerobic glucose limited chemostat) or at an OD600 of 0.3 (anaerobic batch). All batch culture samples were inoculated from overnight pre-cultures to a starting OD600 of approximately 0.01.

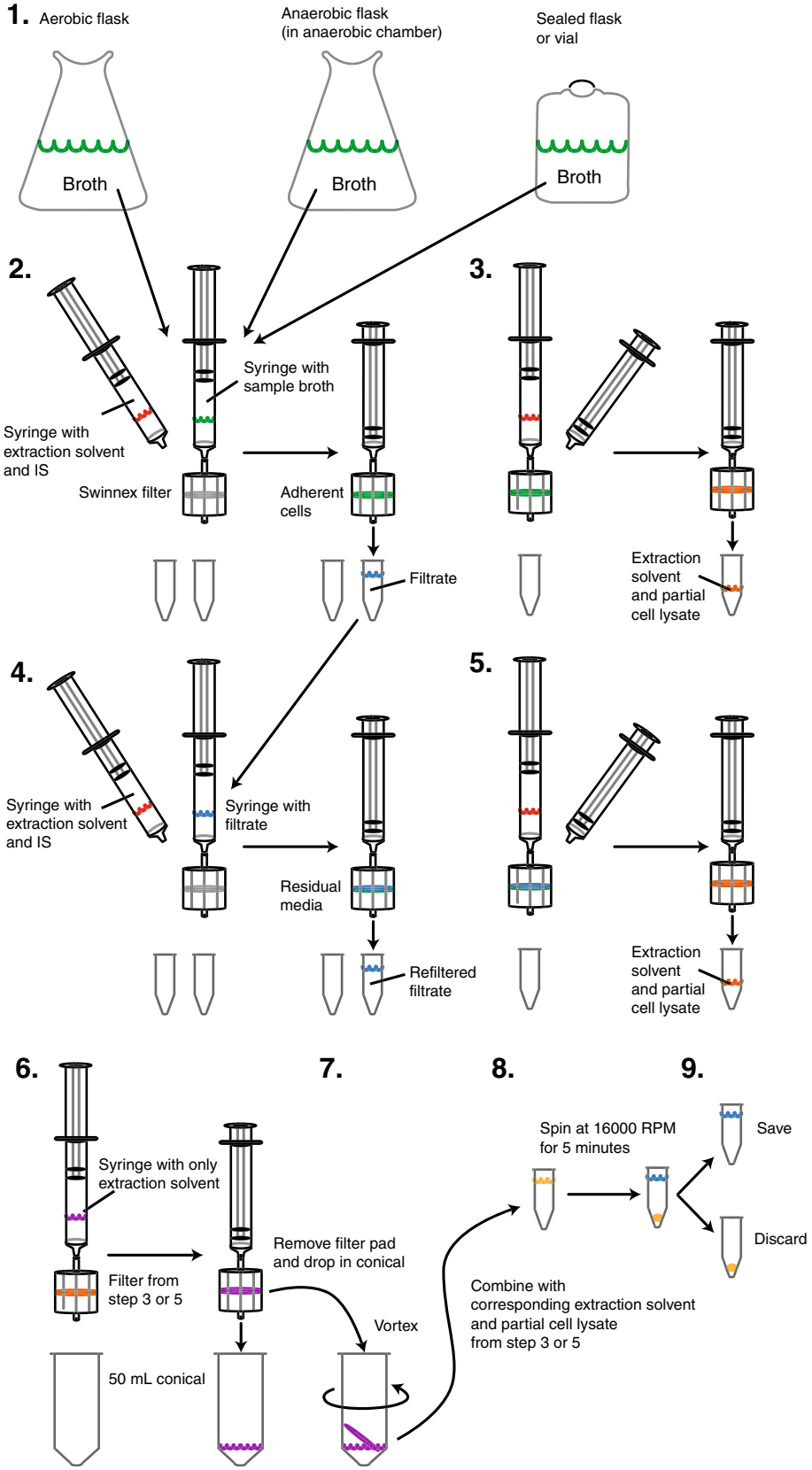
2.3 Sampling and extraction optimization

2.0 mL of culture broth and culture filtrate were sampled and extracted using the FSF approach (Fig. 1) or by direct injection into either pre-cooled organic solvent or liquid nitrogen when specified in the text. The extraction solvents used were 80:20 methanol:water pre-cooled to −80 °C or 40:40:20 acetonitrile +0.1 % formic acid:methanol:water or 40:40:20 acetonitrile:methanol:water with or without buffer (as specified in the text) pre-cooled to −40 °C. The volume of extraction solvent loaded into the syringe was 1.0 mL. For samples taken using the direct extraction approach, the extraction solvent was 4× that of the sample volume for whole broth and filtrate samples. Samples were then serially extracted twice with 200 µL of extraction solvent as described previously (McCloskey et al. 2013).

For anaerobic cultures, a filtrate sample for each replicate was used to calculate the external metabolite concentration. For aerobic cultures without supplementation, a pooled filtrate of replicates was found to be sufficient due to the larger fraction of biomass to media. In addition, it was found that after two rounds of directly extracting the filter and vortexing, subsequent rounds of extraction of the biomass did not improve yields of metabolites. While true for *E. coli*, this would have to be reconfirmed for organisms with a different cellular membrane. The extracts were centrifuged at 16,000 RPM at 4 °C for 5 min. The supernatant was saved and the biomass was discarded. For acidic extraction solvents, the supernatant was neutralized with ammonium hydroxide (8 µL of 1 N ammonium hydroxide per 1 mL of extract containing 40 % acetonitrile +0.1 % formic acid), centrifuged again at 16,000 RPM at 4 °C for 5 min, the supernatant saved and the precipitate discarded. Extracts were evaporated to dryness (Thermo Scientific™ Savant SpeedVac™, Waltham, MA), reconstituted in water, and stored in the −80 °C until analysis. All extracts, extraction solvents, and filter disks contained in filter holders were kept on dry ice between vortexing, centrifugation, and pipetting steps.

2.4 LC–MS/MS analysis and quantification

An XSELECT HSS XP 150 mm × 2.1 mm × 2.5 µm (Waters®, Milford, MA) with a UFLC XR HPLC (Shimadzu, Columbia, MD) was used for chromatographic separation. Mobile phase A was composed of 10 mM tributylamine, 10 mM acetic acid (pH 6.86), 5 % methanol, and 2 % 2-propanol; mobile phase B was 2-propanol. Oven


1.
Aerobic flask
Anaerobic flask
(in anaerobic chamber)
Sealed flask
or vial
Broth
Broth
Broth
2.
Syringe with
sample broth
Syringe with
extraction solvent
and IS
Swinnex filter
Adherent
cells
Filtrate
3.
Extraction
solvent
and partial
cell lysate
4.
Syringe with
extraction solvent
and IS
Syringe with
filtrate
Residual
media
Refiltered
filtrate
5.
Extraction
solvent
and partial
cell lysate
6.
Syringe with only
extraction solvent
Filter from
step 3 or 5
50 mL conical
7.
Remove filter pad
and drop in conical
Vortex
8.
Spin at 16000 RPM
for 5 minutes
Combine with
corresponding extraction solvent
and partial cell lysate
from step 3 or 5
9.
Save
Discard

◀**Fig. 1** Fast filtration sampling and quenching using Swinnex® filters (FSF). *1* An accurate volume of culture broth was either sampled using a pipette and transferred to a syringe attached to a Swinnex® filter with the plunger removed (aerobic cultures) or collected using a syringe and 18.5 gauge blunt needle (anaerobic cultures). For the latter case, the plunger was then extended the volume of the syringe and then attached to a Swinnex® filter. Using a syringe volume that was a minimum of 2× greater than the liquid volume it was to contain allowed for a sufficient gas purge of the filter housing to remove residual culture or filtrate. In practice, we recommend using the largest syringe possible. *2* The cells were separated from the culture broth and retained on the Swinnex® filter pad by rapidly expelling the culture and extra volume gas through the filter housing and into a collection vessel. *3* The syringe was quickly removed, and a second syringe loaded with 1 mL of extraction solvent and labeled biomass pre-cooled to −40 °C was quickly attached to the filter housing. The extraction solvent, labeled biomass, and extra volume gas was rapidly expelled through the filter into another collection vessel. The extraction solvent and partial cell lysate as well as the filter in the filter housing was stored in the −80 °C for further extraction. The same procedure was repeated for each biological replicate. *4* The filtrate from step 2 for each replicate was filtered through a fresh Swinnex® filter, and *5* extracted as in step 3. The Swinnex® filter and extraction solvent were placed in the −80 °C for further extraction. *6* The Swinnex® filter from step 3 or 5 was re-extracted with extraction solvent that does not contain internal standards. The eluent was collected in a 50 mL conical tube. *7* The filter holder was unscrewed over the 50 mL conical so that any residual extraction solvent would not be lost. The filter disk was removed and placed in the 50 mL conical using tweezers. The inside of the filter housing that is attached to the syringe was rinsed with a small volume of the extraction solvent from the 50 mL conical to remove any cells that were detached from the filter disk. The 50 mL conical with extraction solvent and filter disk were then vortexed for 30 s. *8* The extraction solvent and partial cell lysate from step 3 or 5 taken during the sampling procedure were added to the 50 mL conical and vortexed for an additional 30 s. The extraction solvent and cell lysate were then aliquoted into two eppindorf tubes, and the 50 mL conical and filter disk were discarded. *9* The cell debris was pelleted by spinning at 16,000 RPM at 4 °C for 5 min. The supernatant was saved in the −80 °C for analysis and the cell debris was discarded. Further details of the FSF protocol are provided in the supplemental material

temperature was 40 °C. The chromatographic conditions are as follows: 0, 0, 0.4; 5, 0, 0.4; 9, 2, 0.4; 9.5, 6, 0.4; 11.5, 6, 0.4; 12, 11, 0.4; 13.5, 11, 0.4; 15.5, 28, 0.4; 16.5, 53, 0.15; 22.5, 53, 0.15; 23, 0, 0.15; 27, 0, 0.4; 33, 0, 0.4; [Total time (min), Eluent B (vol%), Flow rate (mL min^{-1})]. The autosampler temperature was 10 °C and the injection volume was 10 µL with full loop injection. An AB SCIEX Qtrap® 5500 mass spectrometer (AB SCIEX, Framingham, MA) was operated in negative mode. Electrospray ionization parameters were optimized for 0.4 mL/min flow rate, and are as follows: electrospray voltage of −4,500 V, temperature of 500 °C, curtain gas of 40, CAD gas of 12, and gas 1 and 2 of 50 and 50 psi, respectively. Analyzer parameters were optimized for each compound using manual tuning. The instrument was mass calibrated with a mixture of polypropylene glycol standards.

Samples were acquired using the Analyst® 1.6.2 acquisition software and Scheduled MRM™ Algorithm (AB SCIEX). Integration was performed using MultiQuant™ 2.1.1 (AB SCIEX). IDMS (Mashego et al. 2004; Wu et al. 2005) with metabolically labeled internal standards was used for quantification. In brief, calibration curves of standards spiked with metabolically labeled internal standards were ran with each batch and used to back calculate the analyte levels in the whole broth and filtrate samples. The analyte levels in the samples were scaled to the amount of biomass in each culture determined at the time of sampling by optical density using the conversion factor of cell biomass to cell volume derived by (Volkmer and Heinemann 2011) and experimentally derived conversion of cell density (gDW L^{-1}) to optical density of 0.45 for the used spectrophotometer. The differential method was then applied to the whole broth and filtrate samples (Taymaz-Nikerel et al. 2009) to derive the intracellular concentration. Linear regressions from calibration curves for compound quantification were based on peak height ratios and the logarithm of the concentration of calibrator concentrations from a minimum of four consecutive concentration ranges that showed minimal bias. A peak height greater than 1e3 ion counts and signal to noise greater than 20 were used to define the lower limit of quantification. Quality controls and carry-over checks were included with each batch. Due to the number of biological isomers, the integration of each compound is manually checked.

2.5 Statistical analysis

All statistical and correlation analyses were done using R (R Development Core Team 2011) or MetaboAnalyst (Xia and Psychogios 2009).

3 Results and discussion

A FSF was explored for its suitability for use with anaerobic cultures, and compared to a direct extraction of the whole culture broth and culture filtrate and application of the differential method (Taymaz-Nikerel et al. 2009) (see Sect. 2). An initial comparison between the direct extraction and application of the differential method and fast-filtration methods showed that the number of compounds that can be accurately assayed was increased when using FSF (Fig. 2), but the levels of compounds that turn-over rapidly (e.g., ATP) were decreased, resulting in a low energy charge ratio (Supplemental Figure S1). Therefore, the first priority was to increase the speed at which metabolism was quenched using FSF.

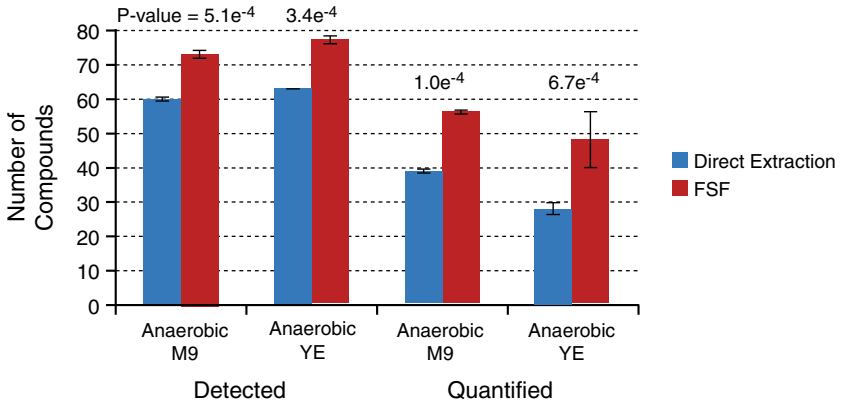


Fig. 2 Sample matrix reduction by FSF. The number of compounds which could be detected with less than 50 % signal contribution from the extracellular medium was higher in FSF samples compared to those obtained by direct extraction of the whole broth and application of the differential method. This was true for both anaerobic, wild-type *E. coli* grown on glucose M9 minimal media and anaerobic, wild-type *E. coli* grown on glucose M9 minimal media supplemented with 1 g L^{-1} of YE. For the data shown, compounds that are considered 'quantifiable' are those that were found to have an average filtrate signal ($n = 3$) of less than 50 % compared to that found for the average intracellular filtration samples ($n = 3$) or the average whole broth direct extraction samples ($n = 3$) (i.e., $\frac{filtrate}{whole\ broth} \times 100 < 50\,\%$). *Error bars* represent standard deviations. The *P* value (two-tailed Student's *t* test) between the direct extraction and FSF are given above the bars

Directly freezing the Swinnex® holder and filter in liquid nitrogen immediately after filtering the culture broth appeared to be a viable strategy to quickly quench metabolism. Unfortunately, cultures sampled using this approach were found to have a physiologically low energy charge ratio (Supplemental Figure S1). When directly extracting the culture broth and filtrate and applying the differential method using organic solvent was compared to freezing the broth and filtrate in liquid nitrogen and then extracting the frozen broth and filtrate with organic solvent, it was deduced that the intermediate step of freezing in liquid nitrogen was insufficient to quench metabolism on the time-scale required to obtain an accurate snap-shot of metabolism. From this finding, a means to expose the filtered biomass to the pre-cooled extraction solvent in a time-frame similar to that of the direct extraction approach was targeted. It was found that by injecting organic solvent into the syringe housing and filter using a second syringe immediately after filtering the culture broth, a dramatic increase in the energy charge ratio could be obtained (Supplemental Figure S1).

The type of extraction solvent used and the addition of buffers to control for pH during the extraction process on compound stability were explored. In agreement with previous findings (Kimball and Rabinowitz 2006; McCloskey et al. 2013), the combination of acetonitrile, methanol, and water was superior to the combination of only methanol and water for the more liable (i.e., phosphorylated) compounds (Supplemental Figure S2). The use of a buffered extraction solvent did not show any improvements in increasing the concentration of the more liable compounds (Supplemental Figure S3). Therefore, acidic acetonitrile was used as the extraction solvent for subsequent tests.

The material of the filter pad utilized in the extraction protocol was varied to understand its impact on the quality of the sample. The comparison of different filter materials revealed that NADH was increased in the cellulose and PVDF filters by 2.2 and 2.1-fold over the PES filters, respectively; NADPH was increased in the cellulose and PVDF filters by 1.2- and 1.4-fold over the PES filters, respectively. The loss of NADH and NADPH in the PES filters could have potentially been due to pi–pi bond interactions between the phenyl group of the PES and the niacin and/or adenine group of the NAD moiety. Also, the oxidized sulfone group could have interacted with the reduced niacin structure. The reduced levels of NADH and NADPH along with several other compounds (i.e., the nucleotide phosphates and glutathiones) with potentials for pi–pi bond interactions resulted in a significant discrimination of the samples taken using the PES filters as determined by a partial least squares discriminatory analysis (PLS-DA) (Supplemental Figure S4). Based on these findings, and the fact that the cellulose filters displayed poor stability in organic solvent, the PVDF filters were used for subsequent tests.

FSF using various syringe sizes was compared to vacuum filtration to ensure that the use of pressure did not affect metabolism prior to extraction. Aside from physical theory, there is empirical evidence that shows that the pressure generated by lower volume syringes can be much greater than larger volume syringes (personal communication with Millipore®). Thus, samples taken using 5, 10, 20, and 60 mL syringes were compared to samples taken

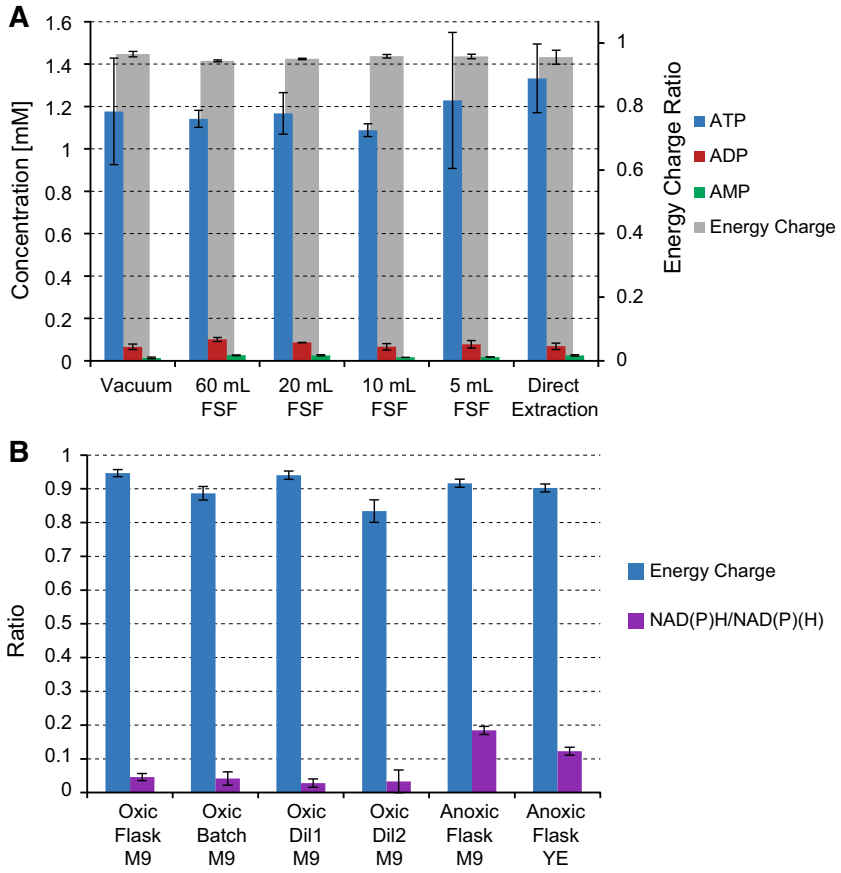


Fig. 3 **a** Comparison of intracellular ATP, ADP, AMP, and energy charge ratio (EC) between aerobic, wild-type *E. coli* grown in glucose minimal media. Cultures were sampled by vacuum filtration, by FSF using 5, 10, 20, and 60 mL syringes, and by direct extraction. Concentrations are averaged values ($n \geq 3$) in units of mM. **b** Comparison of relevant intracellular physiological ratios for wild-type *E. coli* grown in glucose minimal media under aerobic batch growth (Oxic Flask M9), in a bioreactor during batch growth (Reactor Oxic Batch M9), in a bioreactor at two different dilution rates (Oxic Dil1 M9 and Oxic Dil2 M9), and wild-type *E. coli* grown in glucose minimal media under anaerobic batch growth without (Anoxic Flask M9) and with (Anoxic Flask YE) 1 g L^{-1} of yeast extract. Ratios were calculated from average concentration values ($n \geq 3$) in units of mM. *Error bars* represent standard deviations. Energy Charge $= \frac{ATP+ADP/2}{ATP+ADP+AMP}$ and NAD(P)H/NAD(P)(H) $= \frac{nadph+nadh}{nadph+nadh+nadp+nad}$

using vacuum filtration. The effect of syringe volume/pressure was found to be negligible. Indeed, similar levels of AMP, ADP, and ATP were found across all five conditions (Fig. 3). Further, a detailed inspection of all metabolites assayed showed little difference between intracellular levels in each compound class across the different syringe sizes tested (Fig. 4).

Measured differences between the FSF samples and samples taken using the direct extraction method and application of the differential approach for all compounds assayed showed little variation (Fig. 4). Importantly, the energy charge ratio between the filtered and directly extracted samples were approximately equivalent, indicating the speed of quenching metabolism using the optimized fast-filtration was equivalent to that of the direct extraction method (Fig. 3). The coverage of compounds was increased when using the FSF method compared to the direct extraction method (Fig. 4). The levels of more stable compounds (e.g., amino acids) are similar between the FSF method and direct extraction method (Fig. 4). This indicates that the relative recovery of compounds (i.e., ratio of endogenous compounds to metabolically labeled internal standards) between the two methods is equivalent.

The absolute recovery of compounds using the FSF method was tested. The signal intensity of 98 compounds in a neat mixture without any manipulation, after a dry-down in a centrivap, after extraction using the direct extraction method, or after extraction using the FSF method were analyzed (Fig. 5, Supplemental Figure S6). 24.5 % of the compounds were found to have a significant different in signal intensity between either of the groups ($n = 8$, $P < 0.01$, ANOVA). It was found that total signal intensity decreased from the neat mixture without any manipulation (1.18e7, 3.65e7), to the centrivap dry-down

	Vacuum	60 mL FSF	20 mL FSF	10 mL FSF	5 mL FSF	Direct Extraction
Amino Acids						
asn-L	2.72E-01	2.32E-01	1.70E-01	2.75E-01	2.97E-01	
citr-L	2.97E-02	3.51E-02	3.04E-02	3.42E-02	3.15E-02	
glu-L	9.82E+00	8.92E+00	8.15E+00	9.74E+00	9.79E+00	9.37E+00
gln-L	1.94E+00	1.81E+00	1.54E+00	1.98E+00	2.09E+00	2.46E+00
met-L	2.66E-02	2.22E-02	1.92E-02	2.19E-02	1.83E-02	2.00E-02
phe-L	1.77E-02	1.78E-02	1.33E-02	1.59E-02	1.52E-02	1.93E-02
ser-L	1.40E-01	1.62E-01	1.44E-01	1.51E-01	1.91E-01	
thr-L	2.31E-01	1.96E-01	1.98E-01	2.20E-01	2.38E-01	
trp-L	5.63E-03	5.33E-03	4.68E-03	5.57E-03	4.98E-03	5.39E-03
tyr-L	1.75E-02	1.50E-02	1.34E-02	1.67E-02	1.65E-02	2.91E-02
Cofactors and vitamins						
accoa	8.32E-01	8.38E-01	1.05E+00	8.90E-01	7.96E-01	1.19E+00
coa	9.39E-02	9.32E-02	8.23E-02	9.00E-02	9.30E-02	4.83E-02
fad	1.50E-02	1.39E-02	1.65E-02	1.36E-02	1.48E-02	1.36E-02
Nucleosides and bases						
ade	1.32E-03	3.78E-03	2.86E-03	1.70E-03	1.58E-03	
gua	4.42E-04			2.53E-04	3.03E-04	
gsn	3.46E-03	4.20E-03	3.99E-03	3.78E-03	3.68E-03	4.36E-04
hxan	8.16E-05	4.83E-05		3.18E-06		
ins	6.80E-04	9.74E-04	7.22E-04	7.00E-04	6.62E-04	
thym	2.75E-02	4.24E-02	3.58E-02	3.71E-02	3.67E-02	
Organic acids						
akg	5.36E-02	5.22E-02	5.47E-02	6.22E-02	5.60E-02	6.04E-02
5oxpro	1.01E-02	1.81E-02	1.53E-02	1.17E-02	1.28E-02	
acac	1.43E+00	1.54E+00	1.50E+00	1.56E+00	1.50E+00	
acon-C	1.17E-01	2.09E-01	1.73E-01	1.61E-01	1.70E-01	
glutacon	1.41E+00	1.84E+00	1.45E+00	1.53E+00	1.60E+00	
lac-L	2.75E-01			2.55E-01	2.18E-01	5.31E-01
mal-L	7.62E-01	8.17E-01	7.35E-01	7.83E-01	7.94E-01	8.40E-01
cit/icit	3.93E-01			1.90E-01	2.10E-01	
succ	2.53E-01	3.15E-01	2.63E-01	2.63E-01	2.65E-01	
icit	4.94E-03			3.60E-03	3.59E-03	
mmal	3.05E-02	3.84E-02	3.41E-02	3.22E-02	3.26E-02	
phpyr	6.16E-01	8.61E-01	7.44E-01	7.51E-01	7.88E-01	
NAD(P)(H)						
nadh	2.33E-03	7.51E-03	6.15E-03	9.21E-04	1.63E-03	9.03E-03
nadph	4.12E-02	3.54E-02	5.03E-02	4.45E-02	3.20E-02	1.02E-01
nad	2.77E-01	2.54E-01	2.24E-01	2.51E-01	2.44E-01	3.75E-01
nadp	8.51E-02	9.42E-02	7.52E-02	8.16E-02	9.17E-02	9.83E-02
gthrd	7.26E+00	7.73E+00	6.01E+00	7.62E+00	7.39E+00	8.00E+00
gthox	1.62E-01	1.56E-01	1.63E-01	1.59E-01	1.59E-01	3.46E-02

	Vacuum	60 mL FSF	20 mL FSF	10 mL FSF	5 mL FSF	Direct Extraction
Mono- and di-phosphorylated carbohydrates						
23dpg	1.57E-01	1.45E-01	1.29E-01	1.20E-01	9.90E-02	1.50E-01
fdp	8.76E-01	9.01E-01	7.87E-01	9.10E-01	7.05E-01	8.34E-01
6pgc	4.58E-02	1.92E-02	1.62E-02	2.81E-02	3.02E-02	
gam6p	3.84E-02	5.37E-02	5.22E-02	5.40E-02	5.56E-02	6.46E-02
g6p	1.14E+00	9.43E-01	8.35E-01	1.19E+00	1.03E+00	
glyc3p	1.72E-01	2.33E-01	2.08E-01	2.06E-01	2.16E-01	1.37E-01
pep	2.58E-02	3.51E-02	3.58E-02	3.69E-02	3.47E-02	3.25E-02
2pg/3pg	3.49E-01	4.26E-01	3.46E-01	4.29E-01	4.03E-01	1.95E-01
s7p	2.73E-02	2.30E-02	2.17E-02	2.87E-02	2.96E-02	
g1p	1.14E-01	1.48E-01	1.26E-01	1.38E-01	1.68E-01	
dhap	5.21E-01	5.69E-01	5.90E-01	5.97E-01	6.01E-01	3.05E-01
ru5p-D	9.94E-02	1.30E-01	1.23E-01	1.49E-01	1.36E-01	7.66E-02
NDPs						
adp	6.56E-02	1.01E-01	8.59E-02	6.62E-02	7.78E-02	6.82E-02
adpglc	3.30E-03			2.36E-03	2.66E-03	
dadp	5.58E-03	7.67E-03	7.92E-03	5.70E-03	6.52E-03	8.56E-03
dtdpglu	3.08E-03	9.01E-04	2.38E-03		1.99E-03	4.57E-03
gdp	4.51E-02	1.02E-01	1.03E-01	4.28E-02	5.89E-02	6.90E-02
udp	2.88E-02	4.81E-02	3.45E-02	2.38E-02	2.87E-02	3.09E-02
NMPs						
amp	1.33E-02	2.58E-02	2.55E-02	1.61E-02	1.76E-02	2.56E-02
camp	1.16E-02	1.97E-02	1.56E-02	1.60E-02	1.51E-02	
cmp	3.56E-03			2.14E-03	2.90E-03	
damp	2.25E-03	6.31E-03	5.93E-03	3.70E-03	3.58E-03	
dtmp	1.15E-02	1.34E-02	1.24E-02	1.23E-02	1.54E-02	4.57E-03
dump	2.22E-03	2.45E-03	2.57E-03	3.09E-03	3.29E-03	2.45E-03
gmp	7.53E-03	9.58E-03	1.11E-02	8.31E-03	8.03E-03	
imp	2.09E-02	2.10E-02	2.15E-02	2.41E-02	2.01E-02	4.32E-02
NTPs						
atp	1.24E+00	1.27E+00	1.20E+00	1.16E+00	1.35E+00	1.22E+00
ctp	1.52E+00	1.31E+00	1.09E+00	1.03E+00	1.43E+00	3.99E-01
datp	1.68E-01	1.74E-01	1.93E-01	1.82E-01	2.32E-01	
dctp	3.57E-02	2.83E-02	2.79E-02	3.23E-02	3.22E-02	4.61E-03
dttp	9.02E-02	9.05E-02	8.52E-02	8.16E-02	7.29E-02	1.94E-01
gtp	1.49E+00	1.59E+00	1.66E+00	1.49E+00	1.27E+00	
itp	1.34E-01	1.55E-01	1.47E-01	1.50E-01	1.43E-01	
utp	4.04E-01	4.19E-01	3.32E-01	3.46E-01	3.74E-01	3.92E-01

Fig. 4 Heat map comparison of intracellular compounds grouped by compound class for aerobic, wild-type *E. coli* grown in glucose minimal media. Cultures were sampled by vacuum filtration, by FSF using 5, 10, 20, and 60 mL syringes, and by direct extraction and application of the differential method. We found that the metabolite levels for individual compounds are similar between the different approaches. In addition, it is evident that the cultures sampled using vacuum filtration or by FSF allow for quantification of more metabolites than when sampling using the direct extraction method. Metabolite abbreviations are given in Supplemental Table S1. Metabolite levels are based on averages ($n \geq 3$) in units of mM

mixture (1.14e7, 3.57e7), to the direct extraction mixture (1.06e7, 3.46e7), and finally to the FSF mixture (9.54e6, 3.30e7) in both the significantly changed metabolites (Fig. 5), and across all metabolites (Supplemental Figure SF6), respectively. The observed trend correlates with a decrease in signal intensity as the number of sample manipulation steps increased. A likely explanation for this trend is that as the number of sample manipulation steps is increased, a small amount of extracted material is lost. However, because the decrease in signal intensity does not exceed 20 %, minimal affect on acquisition and no discernible effect on quantitation would be expected when using any of the extraction methods used in this study. In addition, the observed trend described above would indicate that sample contamination or degradation is unlikely when using the extraction methods described in this study. Taken together, these results indicate that the final optimized filtration method was able to improve compound coverage and quench metabolism at a rate comparable to the direct extraction method for almost all compounds assayed. The optimized filtration method is shown in Fig. 1.

The suitability of FSF to accurately, reliably, and quickly sample in aerobic or anaerobic conditions from batch or chemostat cultures was investigated by comparing relevant physiological ratios from wild-type *E. coli* samples grown in glucose minimal media (M9). The consistent ratios of energy charge (which would be expected for normal growing cells in glucose media regardless of the culture vessel or availability of oxygen) (Cortassa and Aon

	ST	CE	DE	FSF	P-value	Fisher's LSD
glc-D	3.63E+04	4.98E+04	5.12E+04	1.65E+05	2.70E-06	FSF - CE; FSF - DE; FSF - ST
actp	4.92E+04	3.22E+04	3.33E+04	4.04E+04	6.55E-04	ST - CE; ST - DE
ade	1.36E+06	1.27E+06	1.23E+06	1.02E+06	5.21E-06	CE - FSF; DE - FSF; ST - FSF
akg	8.70E+04	1.07E+05	1.09E+05	1.98E+05	1.21E-08	FSF - CE; FSF - DE; FSF - ST
arg-L	1.41E+06	1.40E+06	1.33E+06	6.42E+05	3.69E-15	CE - FSF; DE - FSF; ST - FSF
asn-L	7.50E+05	7.24E+05	6.58E+05	4.99E+05	7.17E-08	CE - FSF; DE - FSF; ST - DE; ST - FSF
citr-L	4.52E+05	4.54E+05	4.43E+05	3.33E+05	2.01E-05	CE - FSF; DE - FSF; ST - FSF
coa	2.88E+05	2.21E+05	9.24E+04	7.58E+03	9.29E-10	CE - DE; CE - FSF; DE - FSF; ST - DE; ST - FSF
gln-L	8.67E+05	8.50E+05	7.69E+05	6.00E+05	8.00E-08	CE - FSF; DE - FSF; ST - DE; ST - FSF
glutacon	1.05E+05	8.97E+04	9.44E+04	7.85E+05	1.96E-13	FSF - CE; FSF - DE; FSF - ST
gthrd	2.85E+04	1.79E+04	8.60E+03	6.63E+02	5.37E-09	CE - DE; CE - FSF; ST - CE; ST - DE; ST - FSF
gua	1.96E+05	1.95E+05	1.87E+05	1.66E+05	3.86E-03	CE - FSF; ST - FSF
his-L	9.22E+05	9.18E+05	7.54E+05	5.54E+05	6.83E-08	CE - DE; CE - FSF; DE - FSF; ST - DE; ST - FSF
lac-L	9.36E+05	1.07E+06	1.27E+06	1.15E+06	2.98E-03	DE - ST
met-L	8.56E+05	8.26E+05	7.80E+05	6.08E+05	9.15E-06	CE - FSF; DE - FSF; ST - FSF
oaa	6.81E+05	4.21E+05	2.79E+05	3.19E+05	4.39E-08	CE - DE; ST - CE; ST - DE; ST - FSF
orn	2.35E+04	2.30E+04	2.11E+04	7.96E+03	1.45E-19	CE - FSF; DE - FSF; ST - DE; ST - FSF
oxa	2.90E+05	3.19E+05	3.42E+05	5.38E+05	4.63E-12	FSF - CE; FSF - DE; FSF - ST
phpyr	5.30E+04	4.36E+04	4.33E+04	4.70E+04	4.74E-03	ST - CE; ST - DE
pyr	3.03E+04	2.94E+04	2.89E+04	2.37E+04	3.52E-04	CE - FSF; DE - FSF; ST - FSF
ribflv	3.25E+05	2.76E+05	2.05E+05	2.21E+05	2.00E-04	CE - DE; ST - DE; ST - FSF
ser-L	4.12E+05	4.29E+05	4.08E+05	3.13E+05	2.24E-06	CE - FSF; DE - FSF; ST - FSF
succ	2.91E+05	3.22E+05	3.12E+05	4.47E+05	7.77E-05	FSF - CE; FSF - DE; FSF - ST
thr-L	1.32E+06	1.30E+06	1.13E+06	8.56E+05	3.92E-10	CE - DE; CE - FSF; DE - FSF; ST - DE; ST - FSF

Fig. 5 Heat plot of the mean ion count ($n = 8$) for significantly different metabolites ($P < 0.01$; ANOVA) in neat standard mixes that were extracted using different approaches. Neat standard mixes were analyzed without any manipulation (ST), analyzed after a dry-down in a centrivap and reconstituted in water (CE), analyzed after extraction using the direct extraction method (DE), or analyzed after extraction using the FSF method. The reconstitution volume for CE, DE, and FSF was the same as the initial volume of the neat standard mix. The mixes contained 98 representative intracellular metabolites, and were prepared at a concentration of moderate signal intensity for the instrument used. The full table of all 98 compounds is shown in Supplemental Figure S6. Extraction conditions that showed significant differences for a given metabolite (Fisher's least significant difference) are annotated next to the *P* value

1993) were stable across the liquid cultures tested (Fig. 3). The similar ratio of redox equivalents (i.e., NAD+, NADH, NADP+, and NADPH) within aerobic cultures and anaerobic cultures, but differing between aerobic and anaerobic cultures, provides additional evidence that the method can accurately, reliably, and quickly sample from a multitude of culture conditions (Fig. 3). While not explored in this study, the method would be expected to allow for fast sampling from anaerobic bottle cultures, which are commonly used for strict anaerobic cultivation of microbes. The method would also be expected to fare well in the field, where environmental bacterial samples must be obtained.

Finally, in order to assess the impact of compound coverage on knowledge-gained per experiment, the optimized filtration method was applied to investigate the metabolome of anaerobic cultures grown with and without YE supplementation. Whether using FSF or the direct extraction approach, analyses revealed that anaerobic cultures grown with YE supplementation have increased levels of intracellular amino acids and several mono-nucleotide phosphates most likely due to the uptake of amino acids and their precursors from the culture medium (Selvarasu et al. 2009) (Fig. 6). However, the number of amino acids and mono-nucleotide phosphates were higher in the samples taken by fast filtration, and the significant reduction in levels of the Glutathiones and Acetyl-CoA were masked for samples taken by direct extraction.

Twenty-two metabolites were quantifiable (see Fig. 4 caption for cutoff) in both minimal and YE samples using direct extraction, while 81 metabolites were quantifiable in both minimal and YE samples using FSF. For the samples taken by the FSF, 15 compounds were found to be significantly changed between anaerobic growth with and without YE, 6 of which were amino acids and 4 of which were mono-nucleotide phosphates (Fig. 6b). In contrast, for samples taken by the direct extraction approach, only 5 compounds were found to be significantly changed between the two conditions (Fig. 6a). Three of the compounds, including 2 amino acids and NAD+, were common to both sampling approaches. The levels of glutamate and FAD were both found to be changed (increased and

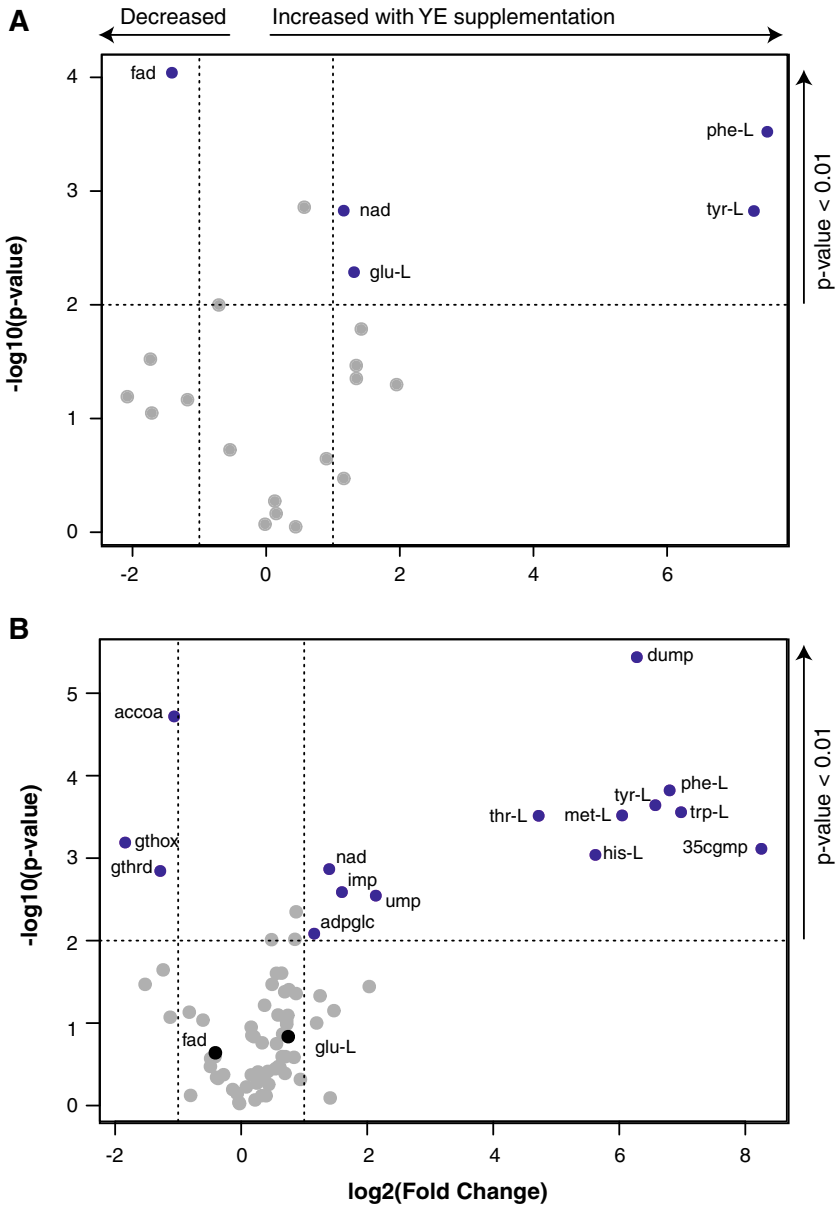


Fig. 6 Volcano plot between wild-type anaerobic *E. coli* cultures grown in 4 g L^{-1} M9 minimal media supplemented with or without 1 g L^{-1} of yeast extract, and sampled by **a** direct extraction or **b** using the optimized FSF method. 22 metabolites were quantifiable (see Sect. 2 for cutoff) in both minimal and yeast extract samples using direct extraction, while 81 metabolites were quantifiable in both minimal and yeast extract samples using FSF. Metabolites with a *P* value greater than 0.01 and fold change greater than 2 are annotated on the plot (shown in *blue*). Out of the five metabolites that met the *P* value cutoff of 0.01 in the direct extraction, three also met this criterion in the fast filtration measurement, and the remaining had *P* values of 0.15 and 0.21 for glu-L and fad, respectively. The *x*-axis reflects the fold change between metabolites between the two conditions (i.e., log2(fold-change)). The *y*-axis reflects the significance (*P* value; two-tailed Student's *t* test) of the changes between the two conditions (i.e., −log10(*P* value)) (Color figure online)

decreased, respectively) in the YE samples compared to the M9 samples for the samples taken by FSF, but they were not found to be significantly changed ($P < 0.01$), nor did they have a fold change greater than two. The other amino acids, oxidized glutathione, ADP-glucose, UMP, dUMP, and 3′-5′-cyclic GMP, could not be quantified in the YE samples for the samples taken by direct extraction. Acetyl-CoA and reduced glutathione were found to be elevated in the M9 samples compared to the YE samples for the samples taken by direct extraction, but their elevation was not significant ($P < 0.01$). The fewer number of compounds that could be detected in the samples taken by direct extraction and the variances in the compounds between replicates resulted in a poorer discrimination between the two groups (Supplemental Figure S5) as determined by PLS-DA. This is exemplified by the change in axis scale between samples taken by fast filtration and direct extraction.

These results indicate that when sampling low biomass cultures supplemented with complex media, the two methods can provide overlapping findings, but the detail and breadth of those findings can be severely decreased if matrix reduction strategies are not employed. The reduced number of compounds and greater variance in the compounds measured when matrix reduction strategies were not employed limited downstream statistical and correlation analysis. The matrix interferences from the culture medium include salts and phosphate buffers that are known to cause ion-suppression (Van Gulik et al. 2012). Besides directly lowering the detection limits via ion-suppression, increased on

column matrix increases the base-line signal noise, which interferes with the detection of low-abundant metabolites. The ability of the fast filtration method to provide a more suitable sample for analysis by LC–MS/MS compared to the direct extraction method allowed for more data points and information to be gained by the same experiment.

4 Conclusion

LC–MS provides a powerful means to analyze intracellular metabolism. Unfortunately, the adverse effects of sample matrix can severely limit the information derived if matrix reduction steps are not included. These steps are a double edged sword in that matrix reduction steps often increase the time it takes to quench metabolism, which results in inaccurate metabolite levels for intracellular metabolites with fast turn-over times (Van Gulik et al. 2012). These difficulties are compounded further when working with anaerobic cultures, where metabolism must be quenched without the introduction of oxygen to the cells.

We have developed, validated, and described a fast filtration method using Swinnex® filter holders termed FSF to overcome these challenges. The method provided fast sampling and quenching to obtain an accurate snapshot of metabolism. The method increased the coverage of compounds that can be detected by reducing matrix interference from the culture medium, which greatly improves the information that can be derived from a given metabolomics experiment. Because the method relies on pressure driven syringe filtration, it is flexible enough to sample anaerobic and aerobic liquid cultures grown in a variety of culturing systems. The developed method was applied to analyze and detail the metabolomes of *E. coli* when growing anaerobically in minimal and complex media containing YE, and key differences were reported. It is envisioned that this sampling modality will provide researchers with a convenient means to obtain accurate intracellular (and simultaneously extracellular if the filtered medium is retained) metabolomics samples beyond those tested in this study. Such samples could include environmental, anaerobic bottles, biofluids (e.g. blood and plasma), and samples from additional culturing conditions.

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Conflicts of interest We have no conflicts of interest.

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