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# Global Metabolic Profiling of *Escherichia coli* Cultures: an Evaluation of Methods for Quenching and Extraction of Intracellular Metabolites

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**Metabolomics and systems biology require the acquisition of reproducible, robust, reliable, and homogeneous biological data sets. Therefore, we developed and validated standard operating procedures (SOPs) for quenching and efficient extraction of metabolites from *Escherichia coli* to determine the best methods to approach global analysis of the metabolome. *E. coli* was grown in chemostat culture so that cellular metabolism could be held in reproducible, steady-state conditions under a range of precisely defined growth conditions, thus enabling sufficient replication of samples. The metabolome profiles were generated using gas chromatography/time-of-flight mass spectrometry (GC/TOF-MS). We employed univariate and multivariate statistical analyses to determine the most suitable method. This investigation indicates that 60% cold (−48 °C) methanol solution is the most appropriate method to quench metabolism, and we recommend 100% methanol, also at −48 °C, with multiple freeze−thaw cycles for the extraction of metabolites. However, complementary extractions would be necessary for coverage of the entire complement of metabolites as detected by GC/TOF-MS. Finally, the observation that metabolite leakage was significant and measurable whichever quenching method is used indicates that methods should be incorporated into the experiment to facilitate the accurate quantification of intracellular metabolites.**

Investigations in microbial metabolomics require reliable and reproducible analysis of the metabolites in the cell across a broad dynamic range of concentrations (nanomolar to millimolar) and from a range of different chemical functionalities. The development of robust and reliable experimental protocols for all steps in the experimental procedure, ranging from biomass cultivation, quenching, and extraction of the metabolome to the quantitative analysis of the metabolites, is required. The analytical methodology available for the detection and identification of metabolites is relatively well developed (see refs 1−4), and these platforms have been optimized.[5,6] More recently the Metabolomics Standards Initiative (refs 7−9 and see http://msi-workgroups.sourceforge.net/) as well as others[3,10−12] have started to discuss the standardization of metabolomics experiments from biological growth−collection through chemical analysis to data processing. However, less consideration has been directed toward optimizing the methods for arresting metabolic activity by quenching protocols and the extraction methods for intracellular metabolites. Villas-Boas et al. have developed optimized quenching and extraction methods for the yeast *Saccharomyces cerevisiae*. However, there are significant chemical and physical differences between the cell envelopes of eukaryotic and prokaryotic organisms, and this means that these protocols may not be directly transferable to prokaryotic organisms. Most notably, the leakage of metabolites during quenching with organic solvents in eukaryotic cells is less severe than with prokaryotic cells,[13,14] demonstrat-

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ing that the integrity of the cell envelope is more readily disrupted in prokaryotic cells.[15]

Global metabolic profiling should seek to meet three criteria: (1) to extract the largest number of metabolites from the cell with suitable recoveries, (2) to prevent exclusion of metabolites as a result of their physical or chemical properties, and (3) to be nondestructive. It is unlikely that one method would enable the detection of the entire cellular metabolome,[13,16,17] due to differing chemistries, viz., polarities, solubilities, and chemical stability. Indeed complementary analytical platforms (e.g., GC/MS, CE/MS, LC/MS, and NMR) would be necessary to give complete coverage of the entire metabolome.[1,18,19] It follows that a multiplicity of extraction methods will also be required to cover the entire range of metabolite physical and chemical properties and to ensure that the metabolites are successfully released from the cell interior and from association with macromolecular structures. Although outside the scope of the present study, the chemical alterations of metabolites at other stages in the sample preparation also need to be carefully considered; by way of example, during trimethylsilylation-based derivatization for GC analysis, glutamic acid is partially converted to pyroglutamic acid by a process of deamination.[20]

The ideal method for metabolite recovery would be easy to perform, robust, and reproducible, and the quenching step should halt metabolism as rapidly as possible without leakage of metabolites from the cell. The aim is to inactivate the intracellular enzymes so that the metabolite profile is "frozen". Speed is of the essence, since a number of metabolic reactions in glycolysis and those concerning ATP involve fluxes of millimoles per liter per second. Quenching of metabolism is usually achieved by a rapid change in temperature or pH. The leakage of metabolites during quenching is likely to be dependent on cell wall and membrane structure, and so an array of procedures is used for different microorganisms,[21] including the yeast *S. cerevisiae*,[13] the Gram-negative bacterium *Escherichia coli*,[17] and the Gram-positive bacterium *Lactobacillus lactis.*[22] Leakage of selected target metabolites containing different functionalities has been reported after contact with 60% aqueous methanol solution, including carboxylic acids and amino acids,[13,23] phosphoenolpyruvate,[24] ATP, and $NAD^+$.[25] The detection of metabolite leakage is undoubtedly biased by the analytical method used,[16] but it is apparent that leakage is unavoidable. Therefore, a quantitative method should be employed to determine the extent of leakage from microbial cells[15] so that the true intracellular metabolite concentrations may be calculated. Minimizing the time in which the biomass is in contact with the quenching solution effectively reduces leakage of cellular metabolites,[13] since organic solvents and cold shock cause leakage of the cell membranes.[23] Therefore, the cells should be separated from the external environment as rapidly as possible, and rapid filtration and centrifugation are commonly employed.

Once metabolism has been arrested by quenching, the metabolites need to be extracted from the cells. The aim here is to permanently deactivate endogenous enzymes and permeabilize the cell to release the maximum number of cellular metabolites with the highest possible recoveries. The target metabolites should not undergo any physical or chemical modification, and degradation should be minimized.[15,16] Although different quenching methods have not previously been evaluated using *E. coli*, Maharjan and Ferenci[17] found that six different extraction protocols produced vast differences in metabolite recovery and using freeze–thaw or boiling to enhance permeabilization of the cell envelope produced further variation. In their study two-dimensional thin-layer chromatography (2D-TLC) was employed to analyze the extracts. However, 2D-TLC only allows for the detection of ~15% of the then proposed metabolites ($n = 791$) of *E. coli* and does not detect low-abundance metabolites.[26] This means that even greater variation might be revealed by more comprehensive mass spectrometric analysis.

The first method of metabolite extraction documented by Dekoning and Vandam[24] was adapted from a total lipid extraction method,[27] providing excellent recovery of phosphorylated compounds. Freeze–thaw cycles or boiling were employed in the extraction methods to facilitate cell permeabilization. However, not all metabolites are stable at high temperatures[17] or extreme pH and some methods have a detrimental effect on some classes of metabolites. The partial destruction of pyruvate, nucleotides, and sugar phosphates was observed during extraction with boiling ethanol.[13,17] There are several disadvantages associated with acid–base extractions. A neutralization step is required, which has a dilution effect on the metabolite concentrations and reduces recovery. The resulting precipitate (e.g., $KClO_4$ from perchloric acid or potassium hydroxide extractions) causes problems during the derivatization procedure for GC/MS analysis and during LC/MS analyses (particularly in the electrospray ionization process), and metabolite quantification may be affected due to absorption of the metabolites to the precipitate.[13] Furthermore, extraction efficiencies were found to be less than 100% by spiking the harvested cell pellet with authentic metabolites,[13] indicating possible degradation during the extraction procedure, or physical losses during sample preparation.

The majority of the above studies focused on a limited number of metabolites rather than attempting to identify all the metabolites present in the biological system. The number of metabolites in *E. coli* is currently documented at 904, with 932 metabolite reactions;[28,29] however, the true number of metabolites is likely


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to increase. This clearly highlights the need for metabolome analysis to allow for the detection of hundreds of metabolites, rather than a selected few, and as such targeted protocols are not suited to global profiling.[13] Therefore, the objective of this study was to produce an optimized method for reliable and reproducible quenching and extraction of the maximum number of metabolites from *E. coli* with the highest possible recoveries. We evaluated three methods of metabolism quenching, which have not previously been tested for *E. coli* cells. We also monitored the supernatant (metabolic footprint, or exometabolome) to evaluate leakage of metabolites during quenching, and we assessed five different extraction protocols. It was essential to ensure that all variations were due to differences in the quenching or extraction procedures rather than variations in cell physiology and metabolism. Therefore, we used chemostat cultures grown under defined nutrient limitations, growth rates, and environmental conditions to ensure the cellular metabolism was held in steady-state conditions throughout the experiment.[30]

## METHODS

**Culture Conditions.** *E. coli* MG1655 was grown in chemostat culture (working volume 490 mL) in a custom-made vessel (750 mL[31]). MS medium was inoculated with overnight culture (25 mL, grown in MSX medium)[32] and grown at 37 °C and pH 6.8 (controlled automatically using a Pierre Gurein (Mauze, France) operating system by addition of 1 M HCl or 1 M NaOH). Aerobic cultures were grown with air sparging at 30 L $h^{-1}$, whereas anaerobic cultures were sparged with 25−30 L $h^{-1}$ $N_2$ and 2.5−3 L $h^{-1}$ $CO_2$. The cultures were mixed with a magnetic stirrer. Once the culture had entered stationary phase, fresh MS medium[33] was fed into the culture vessel at a dilution rate of 0.15 $h^{-1}$ until steady-state was established (five volume changes), and experimental samples began to be taken after a further five volume changes. Glucose-limited cultures were grown with 1 or 2 g $L^{-1}$ glucose with $NH_4Cl$ at 3 g $L^{-1}$, whereas nitrogen-limited cultures contained 0.22 g $L^{-1}$ $NH_4Cl$ and 4.5 g $L^{-1}$ glucose. Anaerobic cultures contained glucose (2 g $L^{-1}$) and excess $NH_4Cl$ (3 g $L^{-1}$).

**Quenching of the Cultures.** Three different methods of quenching the metabolome were assessed in this study and compared with an unquenched, control sample. Samples (12 mg of biomass) were rapidly plunging (<3 s) under pressure into the quenching solution (previously chilled at −48 °C in a standard 28 mL bottle, Fisher Scientific, U.K.), which was immediately connected to the sampling port prior to sampling. Samples were maintained on ice throughout all manipulations, and five replicate samples were taken for each analysis.

*Biomass Quenching.* Culture samples (10 mL) were plunged rapidly into an equal volume of either 60% aqueous methanol solution (−48 °C), tricine-buffered (0.5 mM, pH 7.4) aqueous 60% methanol solution (−48 °C), or boiling absolute ethanol (90 °C). The quenched biomass was centrifuged for 8.5 min (minimum time period required to pellet biomass) at 3000*g* and −9 °C. The supernatant was removed rapidly, and an aliquot (1 mL) was retained to assess the leakage of internal metabolites. The pellets and supernatants were snap frozen in liquid nitrogen and stored at −80 °C for further analysis. Although when employing ethanol it is usual to perform the metabolite extraction immediately, we chose to store this so as to perform metabolite extractions at the same time to avoid variability.

*Control Sample.* Culture was rapidly removed to a prechilled standard 28 mL bottle (−48 °C) and then transferred to a prechilled centrifuge tube and centrifuged for 8.5 min, 3000*g*, at −9 °C. The retained biomass (cell pellet) was snap frozen in liquid nitrogen and storied at −80 °C for further analysis.

*Footprint Analysis.* An aliquot of culture (1 mL) was rapidly filtered through a syringe filter (0.22 $\mu$m; Millipore express) to remove the cells. The filtrate was snap frozen in liquid nitrogen and stored at −80 °C for further analysis.

**Extraction of Metabolites.** Five methods of metabolite extraction were investigated; these methodologies were modified accordingly from Maharjan and Ferenci[17]. All extraction methods were compared against the four quenching methods described above (i.e., 20 experiments). Five replicate extractions were performed for each method (i.e., 100 samples).

*Methanol Extraction.* The biomass pellets were suspended in 500 $\mu$L of 100% methanol (−48 °C), frozen in liquid nitrogen, and allowed to thaw on dry ice. The freeze−thaw cycle was performed three times in order to permeabilize the cells, resulting in the leakage of the metabolites from the cells. The suspensions were centrifuged at 16 060*g*, at −9 °C, for 5 min. The supernatant was retained and stored on dry ice, and a further aliquot (500 $\mu$L) of 100% methanol (−48 °C) was added to the pellet. The above procedure was repeated, and the second aliquot of methanol was combined with the first. The sample was stored on dry ice for further analysis.

*Methanol/Chloroform Extraction.* The pellets were suspended in 1 mL of freshly prepared methanol/chloroform solution (2:1). The freeze−thaw cycles were performed as outlined above, and 250 $\mu$L of ice-cold 0.5 mM aqueous tricine was added to the solution. Tricine is considered to be the most suitable buffering system for mass spectrometry applications.[34] The samples were mixed, and the different phases were allowed to separate; the upper methanol phase was removed and stored on ice. A further aliquot (250 $\mu$L) of tricine was added to the sample, and the phase separation procedure was repeated. The upper methanol layer was added to the first, and the solution was centrifuged at 16 060*g*, at 0 °C, for 5 min to remove any cell debris. The supernatant was transferred to a fresh tube, and the samples were stored on dry ice for further analysis.

*Perchloric Acid Extraction.* An aliquot (1 mL) of ice-cold 0.25 M perchloric acid (PCA) was added to the biomass. Three freeze−thaw cycles were performed on the resuspended biomass as described above. The sample was centrifuged to remove the biomass from the samples, and the supernatant was neutralized with KOH. The resulting precipitate ($KClO_4$) was removed by centrifugation, and the supernatant was stored on ice for further analysis.

*Boiling Ethanol Extraction.* An aliquot (1 mL) of boiling absolute ethanol (90 °C) was added to the pellet, and the solutions

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were heated in a closed tube for 10 min at 90 °C. The samples were cooled on ice for 5 min, and the cell debris was removed by centrifugation. The retained supernatant was stored on dry ice for further analysis.

*Potassium Hydroxide Extraction*. The biomass was resuspended in 1 mL of 0.25 M KOH (80 °C), and the samples were heated at 80 °C for 10 min and then cooled on ice for 5 min. The cell debris was removed by centrifugation, and the supernatant was neutralized with PCA. The samples were centrifuged to remove the resulting precipitate ($KClO_4$), and the supernatant was stored on ice for further analysis.

**Generation of Metabolome Profiles.** *Sample Preparation for GC/MS Analysis*. Aliquots (700 $\mu$L) of each extract was spiked with 100 $\mu$L of internal standard solution (0.19 mg mL$^{-1}$ succinic-$d_4$ acid, 0.27 mg mL$^{-1}$ malonic-$d_2$ acid, 0.22 mg mL$^{-1}$ glycine-$d_5$ in HPLC-grade water) and lyophilized (HETO VR MAXI vacuum centrifuge attached to a HETO CT/DW 60E cooling trap; Thermo Life Sciences, Basingstoke, U.K.). Samples were subsequently derivatized in two stages. An aliquot (40 $\mu$L) 20 mg/mL *O*-methylhydroxylamine solution in pyridine was added and heated at 40 °C for 80 min followed by addition of 40 $\mu$L of MSTFA (*N*-acetyl-*N*-(trimethylsilyl)-trifluoroacetamide) and heating at 40 °C for 80 min. A retention index solution was added for chromatographic alignment (20 $\mu$L, 0.6 mg mL$^{-1}$ $C_{10}/C_{12}/C_{15}/C_{19}/C_{22}$ *n*-alkanes).

*Gas Chromatography/Time-of-Flight Mass Spectrometry Analysis.* The gas chromatography/time-of-flight mass spectrometry (GC/TOF-MS) method used to analyze the metabolites is suitable for detection and semiquantification of a wide range of metabolite classes (in this study 264 distinct derivatized peaks were detected) including amino acids, organic and fatty acids, sugars and sugar alcohols, amines, and many other metabolites as detailed in available mass spectral/retention index libraries (author's libraries and Golm metabolome database (http://csbdb.mpimp-golm-.mpg.de/csbdb/dload/dl_msri.html)) or genome-scale reconstructions of metabolic networks.[19] Samples were analyzed in a random order employing GC/TOF-MS (Agilent 6890 GC coupled to a LECO Pegasus III TOF mass spectrometer) using the optimal settings determined previously for yeast analysis.[5] Raw data were processed using LECO ChromaTof v2.12 and its associated chromatographic deconvolution algorithm, with the baseline set at 1.0, data point averaging of 3, and average peak width of 2.5. A reference database was prepared, incorporating the mass spectrum and retention index of all metabolite peaks detected in a random selection of samples (one sample per quenching or extraction class) so to allow detection of all metabolites present, whether or not expected from the study of bibliographic data. Each metabolite peak in the reference database was searched for in each sample, and if matched (retention index deviation less than ±10; mass spectral match >750), the peak area was reported and the response ratio relative to the internal standard (peak area-metabolite/peak area-succinic-$d_4$ acid internal standard) calculated. These data (matrix of $N$ samples × $P$ metabolite peaks) representing normalized peak lists were exported in ASCII format for further analysis.

**Multivariate Data Analysis.** Many multivariate data reduction and analysis techniques are available for studying metabolomic data sets (e.g., refs 35−37). Principal components analysis[38] is an unsupervised technique which assumes no a priori knowledge of class structure (in this study a combination of different growth conditions and extraction methods) and acts to reduce the dimensionality of multivariate data while attempting to preserve as much of the cross-dimensional variance as possible. Principal components analysis was used to look for clusters indicating the similarity of specific samples. Before multivariate analysis the GC/MS data (345 peaks × 200 samples) was mean-centered and Pareto-scaled (columnwise) so that each $m/z$ variable is directly comparable.

## RESULTS AND DISCUSSION

**Growth of *E. coli* in Chemostat Culture.** Samples of *E. coli* were produced for metabolome analysis by growing the cells in a chemostat to ensure that the cell physiology was well defined and reproducible. A chemostat culture was established under the first glucose limitation; the growth conditions (reduced glucose limitation, nitrogen limitation, and anaerobic conditions) were sequentially altered to perturb the cell physiology and, thus, produce changes in the metabolite profile. At the end of the experiment the culture was restored to the starting glucose limitations to ensure the selection of any mutations did not induce metabolic changes.

**Effect of Quenching Methods.** Three protocols were evaluated to determine the most suitable method to quench metabolism in the cells (Figure 1). A control sample and footprint analyses were also included in the experimental design to aid in the assessment of leakage of intracellular metabolites into the quenching solution. The control tube employed simple chilling at −48 °C and was used as a guide to the number of peaks detected by each extraction method (as shown in Figure 1) rather than an effective means of quenching the metabolism. Figure 1 illustrates the number of peaks for each method, represented as a percentage of the total number of peaks detected in the experiment. It should be noted that the chemical derivatization procedure (*O*-methylhydroxylamine and MSTFA) may react with more than one reactive functional group per metabolite, and this may artificially inflate the number of metabolites detected. The largest number of peaks (152 and 154) was observed when the cells were quenched with either 60% aqueous methanol or 60% aqueous methanol/tricine solution. Although the addition of tricine did not appear to have a beneficial effect on the majority of the methods, the number of peaks detected in the KOH extractions was higher when tricine was included in the quenching solution. In a previous study on eukaryotic microbes, the addition of buffer was not found to be beneficial in reducing metabolite loss during the quenching of metabolism.[13] There was not a noticeable difference in the peak numbers observed between the chilled universal (control) and the methanol and methanol/tricine quenching methods. The employment of hot ethanol as a quenching solution had a detrimental effect on the number of peaks detected in the majority of the methods. This may have been due to degradation of


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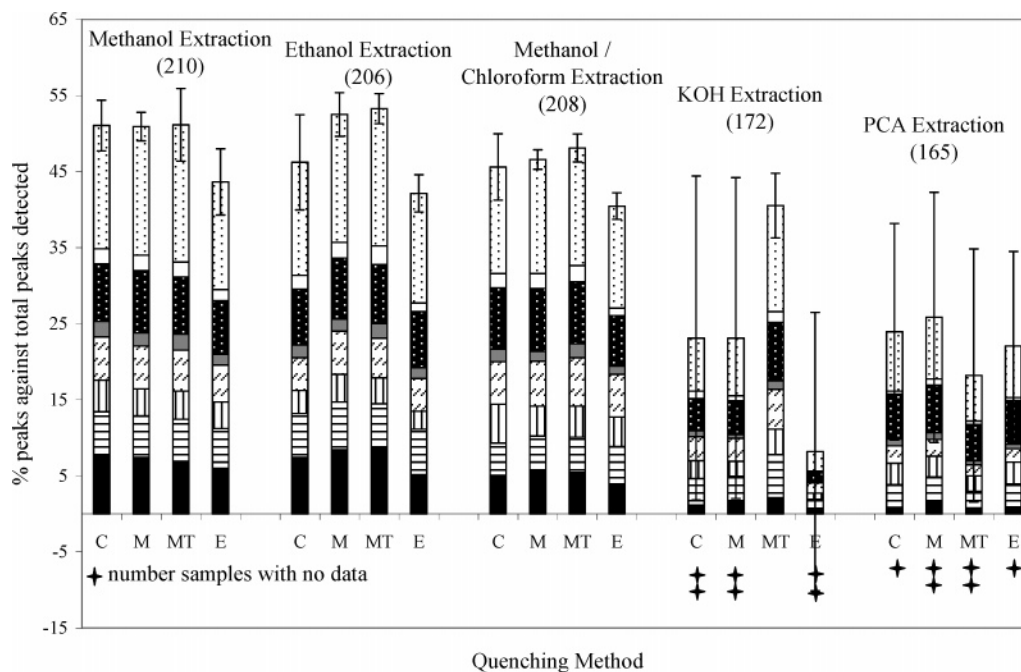


**Figure 1.** Recovery of derivatized peaks from *E. coli* cells to assess the three quenching (control (C), 60% methanol (M), 60% methanol with 0.5 mM tricine (MT), and ethanol (E)) and five extraction procedures. The analyses were performed on biomass cultured under glucose limitation (2 g $L^{-1}$). The data is represented as a percentage of the total number of peaks (264) detected across all five extraction methods, and the total number of peaks detected by each extraction method is shown in parentheses. The error bars represent the standard deviation of the total numbers of peaks detected in each method. The derivatized peaks were classified according to chemical classes: unknown, black spots; sugar phosphates, white; sugars and sugar alcohols, white spots; phosphate derivatives, gray; organic acids, diagonal lines; others, vertical lines; fatty acids, horizontal lines; amino acids and nitrogen-containing compounds, black. The crosses indicate the absence of replicates in the acid and base extractions.

thermolabile metabolites or an increase in the cell permeability and leakage of metabolites.[25] The large error observed for the acid and base extractions (Figure 1) was due to the high salt content (as a result of the neutralization step) which interfered with the derivatization procedure.

A quantitative measure of the efficiency of metabolite recovery is required. One approach is to spike a metabolite mix into the extraction solution to calculate analytical recoveries.[13,39] However, this only assesses recovery from a free solution state, and we feel this is an inappropriate method to calculate metabolite recovery from the intracellular volume. Direct measurement of metabolite recovery from the intracellular volume is fraught with difficulty, since it would involve ensuring that the cells take up equivalent concentrations of a reference metabolite, and it would be difficult to achieve in a reproducible manner. The approach that we propose is to use a set of chemically identified metabolites which represent a variety of different chemical classes and are found in the majority of analyses (>75%). By measuring the detector response for each of these metabolites in the samples and comparing these with the response for the internal standard, it is possible to calculate relative recoveries. In this way, we were able to produce a quantitative analysis comparing the effect of the quenching solutions on the relative recovery of the metabolites (Figure 2). It is apparent from this analysis (Figure 2) that contact with solvents during the quenching step decreased the relative recovery of a number of these metabolites, including putrescine and glutamic acid/pyroglutamic acid. This was accompanied by an increase in the amount of these metabolites released into the quenching solutions as determined by comparison with the footprint analysis. In agreement with the previous observations, quenching with 60% methanol produced the greatest recovery of the majority of the selected metabolites, whereas boiling ethanol quenching decreased the recovery.

**Leakage of Metabolites during Quenching of Metabolism.** Leakage of intracellular metabolites was assessed by comparing the metabolites detected in the quenching supernatants to those in the footprint samples. It was observed that 65 metabolites were present in the quenching supernatants but not the footprint samples (Figure 3), indicating the leakage of the metabolites during the quenching procedure. Such leakage will inevitably affect the accuracy of quantification of intracellular metabolites. Therefore, the relative response of each metabolite detected in the quenching supernatants was compared to that in the footprint samples. The greatest number of metabolites (107) which increased in the quenching supernatants relative to the footprint samples was detected during quenching with hot ethanol. The numbers were slightly lower for methanol and methanol/tricine with 95 and 97 peaks showing an increase in quenching solutions. The relative response of each metabolite was also compared to the control samples. This process was performed for each extraction procedure to account for any variation in the methods. The metabolite was scored if the response was observed to decrease in relation to the control, thereby indicating leakage (Table 1). In accordance with the above observations the leakage was more pronounced during ethanol quenching. There was no

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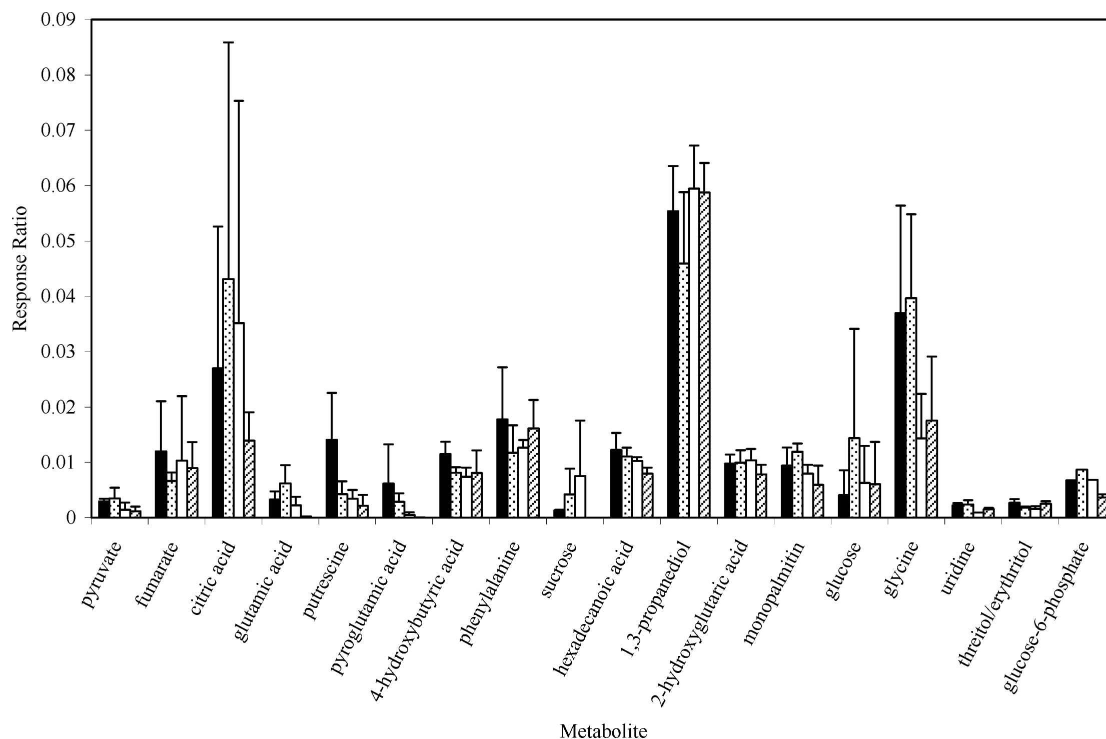


**Figure 2.** Quenching efficiency: the relative recovery of a number of representative metabolites from different chemical classes was compared by dividing the peak area by the peak area of the internal standard to illustrate the effect of the quenching regimes. The analysis was performed on metabolite profiles generated from cells grown under glucose limitation (2 g $L^{-1}$), and the metabolites were extracted using methanol. The response ratio of pyroglutamic acid and putrescine was divided by 100, and that of hexadecanoic acid was divided by 10 to aid visualization of the data. The quenching methods are illustrated as follows: black, control; black dots, 60% methanol; white, 60% methanol and 0.5 mM tricine; diagonal lines, ethanol.

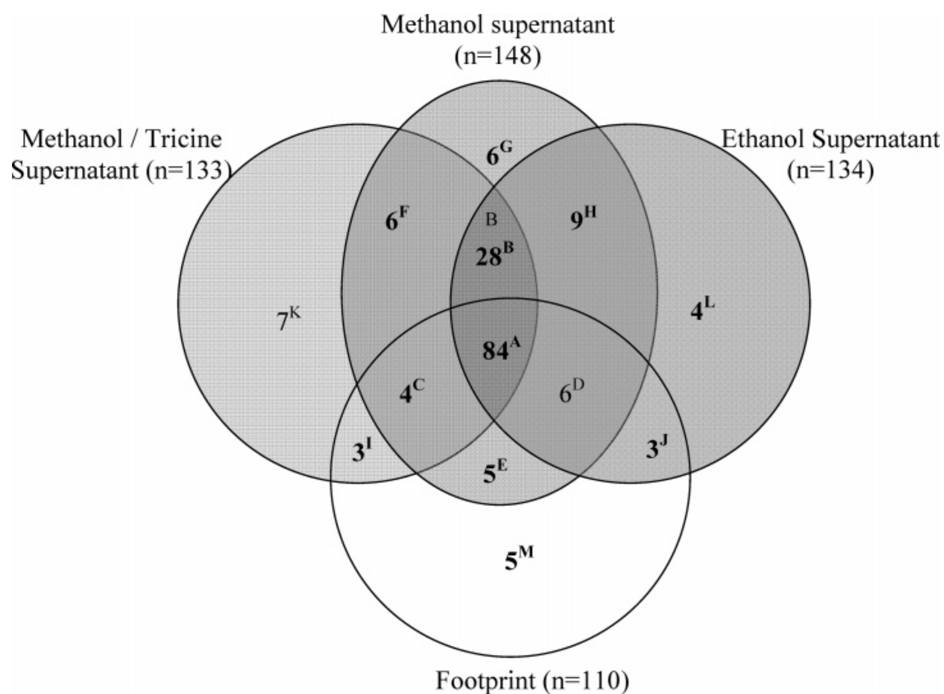


**Figure 3.** Venn diagram illustrating the detection of derivatized peaks ($n$ = 170) in the supernatants of the quenching solutions and footprint samples. One peak was detected in the footprint samples, methanol/tricine, and ethanol supernatants; however, it was not possible to include a separated section to include this on the Venn diagram; it was therefore counted in the footprint and ethanol supernatant section. Identification of the metabolites from each sector (indicated by A–M) are illustrated in Supporting Information Table 1.

significant difference between quenching with 60% methanol solution or 60% methanol and 0.5 mM tricine, except during the combination of methanol/tricine quenching and KOH extractions. The findings indicate that quenching with hot ethanol should only be applied when a combined quenching–extraction procedure is employed.

**Table 1. Comparison of Metabolites Detected Across the Various Methods in Relation to the Quenching Control[a]**

| quenching method | extraction method | | | | |
|---|---|---|---|---|---|
| | methanol | ethanol | methanol/ chloroform | KOH | PCA |
| methanol | 96 | 92 | 117 | 52 | 106 |
| methanol/tricine | 114 | 93 | 96 | 35 | 109 |
| ethanol | 139 | 126 | 112 | 128 | 102 |

[a] The relative recovery of each metabolite was subtracted from the corresponding quenching control. The metabolite peak was scored if the response was observed to decrease in relation to the control; the table illustrates the total number of peaks detected.

Previous studies have demonstrated that leakage of metabolites occurs during quenching of metabolism with organic solvents; however, few studies have quantified the extent of the leakage (see the review in ref 15). The quenching of *S. cerevisiae* cells with cold methanol was reported to damage the cell integrity permitting leakage of the metabolites,[13] and it was estimated that 5% of the yeast population would be permeabilized during quenching with methanol (−40 °C).[16] It is technically problematic to quench metabolism effectively without leakage of metabolites from the cells. Filtration of the cells is one method to reduce leakage;[21] however, this is only suitable for metabolites with a turnover in the order of minutes to hours and when low biomass concentrations are adequate. The time requirement and technical demands of filtering 12 mg of biomass required for global profiling would not permit the use of this technique as an effective method. Although the use of an isotonic quenching solution (0.9% w/v saline solution or 0.85% w/v ammonium carbonate solution[40]) may reduce leakage of metabolites,[21,23] it is not possible to maintain the cells below the required −20 °C prior to the extraction of the metabolites;[39] moreover, salts are not MS friendly as in ESI they generate adducts and can cause matrix effects. The residence time of cells in organic solvent has reportedly had an influence on the degree of metabolite leakage. Very rapid centrifugation is preferable, but this is not technically possible as very high *g*-forces will result in cell permeabilization.

**Effect of Extraction Methods.** *Assessment of Global Methods.* The organic solvent extractions produced the greatest number of detected peaks, with the methanol and ethanol methods yielding the highest numbers (Figure 1, Supporting Information Figure 1). This observation is consistent with other studies investigating metabolite extractions in *E. coli* and *S. cerevisiae*.[13,17] There was a slight reduction in the peak numbers when methanol and chloroform were employed to extract the metabolites (Figure 1). The solubility of metabolites is related to their polarity, such that the more polar extraction solutions will dissolve more polar compounds and vice versa. Thus, the reduction in the numbers of peaks was probably due to partitioning of the fatty acids and lipids into the chloroform phase (which was not analyzed in this study). The methanol/chloroform method is also more technically demanding, time-consuming, is not easily automated, and may be more prone to experimental errors. This method would not be ideally suited to global high-throughput analyses, and as such we would not recommend the use of methanol/chloroform for global analyses unless the extraction-based separation and detection of lipids is necessary. Our findings were in agreement with a previous study.[13] In previous studies, methanol/chloroform extractions were reported to be successful for the extraction of specific metabolites which may be problematic to extract by other methods, for example, phosphorylated compounds.[22,41] However, our findings did not support this observation (Figure 4 and Supporting Information diagram 1). To illustrate the overlap of the metabolites extracted by the five different methods a Venn diagram (Figure 5) was constructed representing all the extractions ($n$ = 100) performed on the first glucose-limited culture condition. This superset analysis illustrates that a total of 264 derivatized metabolite peaks were detected when the methods were combined and a high number (104) were represented in all of the five methods. The highest number of peaks (207) detected by a single method was observed in the methanol extractions, with similar peak numbers (201 and 202) observed in the ethanol and methanol/chloroform extractions. The PCA and base (KOH) extractions yielded low numbers of peaks and very poor reproducibility. A neutralization step is required prior to analysis, and the resulting precipitate is difficult to remove, thereby increasing the salt concentration in the sample. This increased the mass of solid residue after lyophilization, which interfered with the efficiency of derivatization for GC/MS analyses. Our findings agree with a previous study,[13] which suggested that the methods were not suited to global metabolome profiling. However, the KOH extractions yielded the most complementary information to the methanol-extracted profiles with the detection of 37 peaks not recovered in the methanol method (Figure 5). The greatest number of unique peaks (12) was detected in the KOH method, in which the majority were short-chain organic acids (Supporting Information Table 2). This may indicate that the extraction solution pH may influence the extraction of basic and acidic metabolites.

*Multivariate Data Analysis.* The data generated in this investigation are extremely complex, and it is not adequate to apply univariate data analysis alone. Therefore, the multivariate method of principal components analysis was employed to investigate reproducibility of the extraction procedures. To illustrate this, the first two principal components (PC) which account for 68.8% of the variance in the data (the entire selection of metabolites) are plotted against each other (Figure 6). The proximity of the replicate samples ($n$ = 5) in the scatter plots reflects the reproducibility of the extraction procedure and growth condition. Chemostat culture was used to minimize the metabolic variation in cells within the same growth condition. In the analysis of the profiles generated by the methanol extraction method (Figure 6a), a general trend was observed across PC1 and PC2 separating the glucose-limited cells from the samples cultured under anaerobic and nitrogen-limited conditions. From a biological perspective, the shift in metabolism between the cells cultured under the glucose limitations and those grown under nitrogen-limited or anaerobic conditions would be expected to produce the largest difference. Therefore, the analysis performed on the methanol-extracted cells demonstrates that the observed variance is a direct reflection of the biological variation in the data set. By contrast, the analysis

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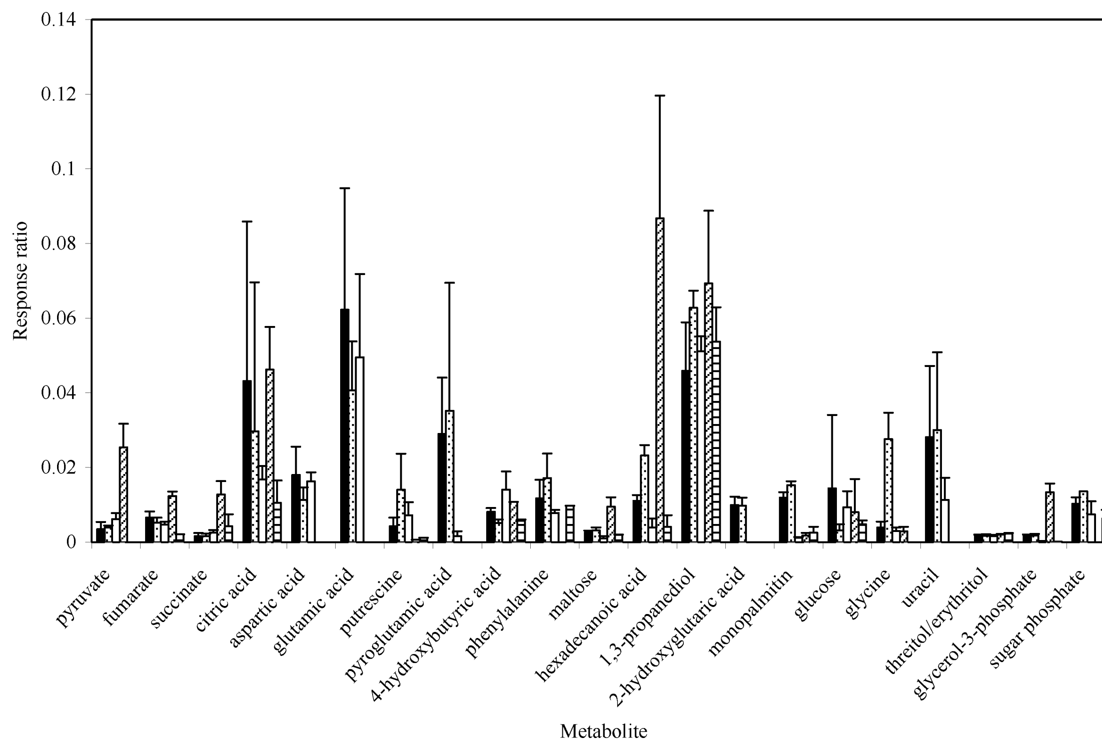


**Figure 4.** Extraction efficiency: the relative recovery of a number of representative metabolites from different chemical classes was compared by dividing the peak area by the peak area of the internal standard to illustrate the effect of the different extraction regimes. The analysis was performed on biomass cultured under glucose limitation (2 g $L^{-1}$); the biomass was quenched with 60% methanol solution. The response ratio of putrescine and glycerol-3-phosphate was divided by 100, and that of citric acid, succinate, glutamic acid, and pyroglutamic acid was divided by 10 to aid visualization of the data. The different extraction methods are illustrated as follows: black, methanol; black dots, ethanol; white, methanol/chloroform; diagonal lines, KOH; horizontal lines, PCA.

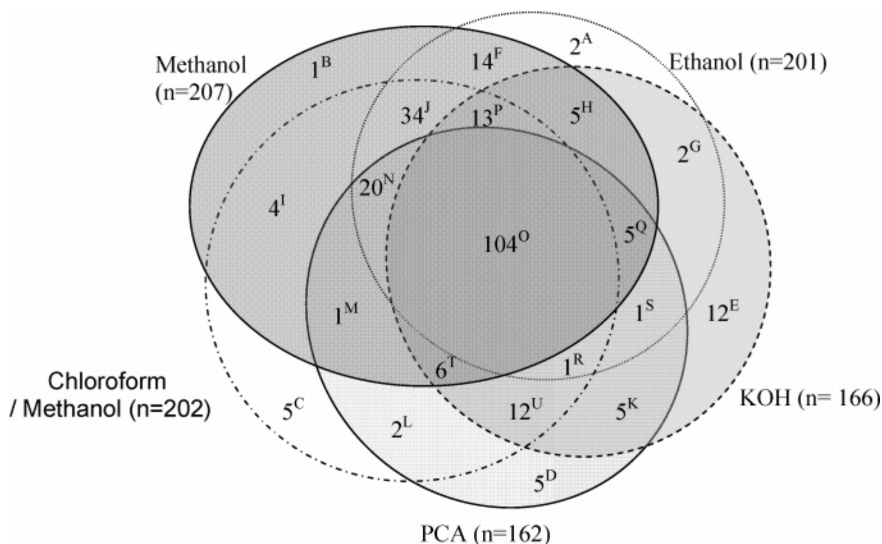


**Figure 5.** Venn diagram illustrating the distribution of peaks ($n$ = 264) from the five different extraction methods performed on quenched biomass from the first glucose limitation (2 g $L^{-1}$). The Venn diagram summarizes 100 independent extractions. Five peaks were not included in the diagram due to constraints of the Venn diagram; the methods available to extract these metabolites are illustrated in Supporting Information Figure 2 along with identification of the metabolites identified in each section of the Venn diagram.

of the samples extracted by PCA did not show trends related to biological significance (Figure 6b). The replicate samples from cells cultured under the same growth conditions did not cluster together, except for the replicates grown with 1 g $L^{-1}$ glucose. The greatest difference represented in the data occurred between the latter samples and the rest of the samples, which suggests that the coclustering is fortuitous and does not imply any biological significance.

**Efficiency of Metabolite Extraction Protocols.** The relative recovery of a subset of the analyzed metabolites was used to investigate the efficiency of the five extraction methods (Figure 3). The analysis illustrates that metabolite recovery is greatly influenced by the method of extraction, and one particular method does not produce the greatest recovery of all the selected metabolites. The three organic solvent extractions appeared to produce the most consistent recovery, with methanol and ethanol

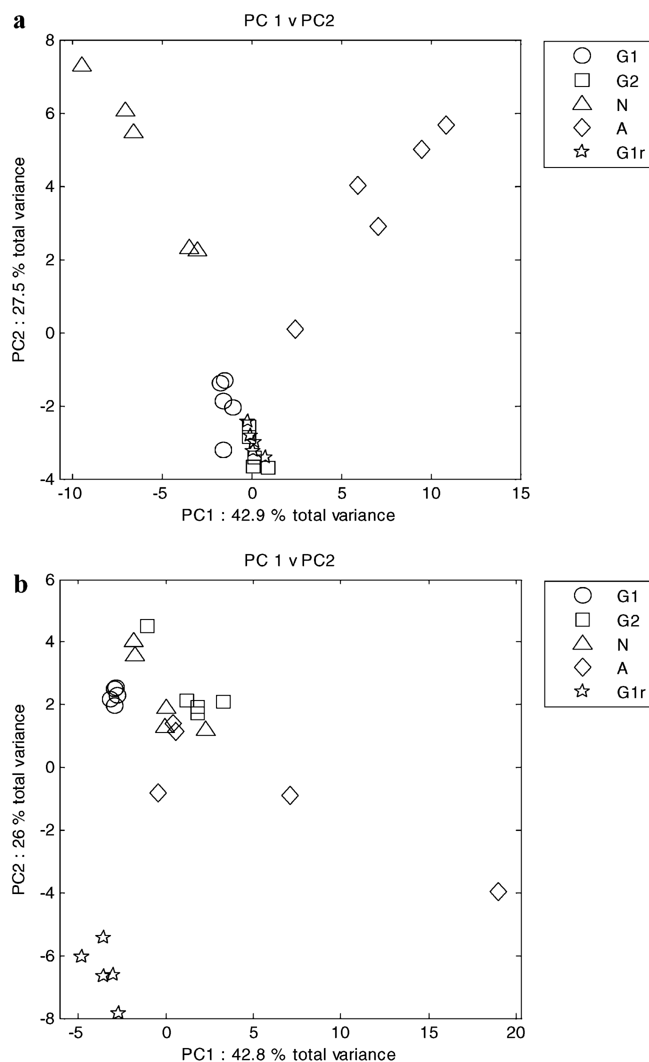


**Figure 6.** Principal component analysis of the metabolome profiles from the five growth conditions extracted by (a) methanol and (b) perchloric acid.

being the most useful. There are specific examples when a particular method may not be suitable for a specific metabolite/metabolite class; for example, 2-hydroxyglutaric acid was not detected in the methanol/chloroform method, and the peak area for pyroglutamic acid/glutamic acid was considerably reduced in relation to the other organic solvent methods. The detection of sugar phosphates was reported to be problematic with methanol/chloroform extraction due to reduced solubility.[17] In our investigation the low recovery of glycerol-3-phosphate and an unknown sugar phosphate in comparison to the other organic solvent methods is consistent with this observation. A reduction or absence of the majority of the metabolites was noted with the PCA extractions, whereas the KOH method had more variable results in respect of number of peaks detected and relative response. Thus, the peak areas for some metabolites (including glycolysis and TCA cycle intermediates) were higher than those obtained with other extraction methods employed, whereas others, including aspartic acid, glutamic acid, and putrescine were lower. The detection of sugar phosphates and sugars was previously reported to be problematic with KOH extractions.[13] Our results exhibit slightly conflicting results, in that an unknown sugar phosphate is not recovered by KOH extraction but the relative response for glycerol-3-phosphate is considerably higher than in the other methods.

Although some extraction methods are not ideally suited to global analyses, they may have applications when the detection of a specific metabolite or metabolite class is required. It has previously been suggested that extraction protocols should be classified in order to target classes of metabolites;[15] we have indicated some general suggestions in this paper and provided information relating to the detection of specific metabolites (Figures 2 and 4, Supporting Information Figure 1, Supporting Information Tables 1 and 2).

## CONCLUSIONS

The objective of this investigation was to develop a standard method for the quenching and extraction of metabolites in *E. coli* for global metabolite analysis. Different quenching methods have not previously been evaluated for *E. coli* cells, and investigations into the quenching of other microorganisms have only focused on variations on methanol quenching protocols. The leakage of metabolites during the quenching procedure has been demonstrated in other studies, although few have attempted to quantify the amount. Our findings confirm that leakage does occur for *E. coli*. By monitoring the metabolites in the quenching solution and in footprint samples, we have demonstrated that leakage of metabolites is more pronounced during hot ethanol quenching. The presence of a buffering system, based on tricine, does not appear to have a beneficial effect during methanol quenching of *E. coli* cells.

While previous studies have investigated different methodologies for the extraction of metabolites from microbial cells, they generally involve only a limited number of metabolites, and this means that they are not necessarily suited to global analyses. The assessment of the extraction procedures in our study showed that the organic solvent methods were the most suitable for one-step global analysis, with the methanol and ethanol methods resulting in the highest peak numbers. The alcoholic solvent methods are effective in the extraction of metabolites because they denature and precipitate proteins and polysaccharides, do not warrant the addition of salt which is problematic in mass spectrometry studies, are easy to evaporate/concentrate, and result in minimal pH effects.[17] The multivariate analyses on all extraction methods indicates that the suite of methanol extractions appear to be the most reproducible and would therefore be ideally suited as a global method.

Although some methods are not suited to global analyses, they may be useful for targeted analyses, for example, the extraction of organic acids with KOH. The Supporting Information data shows the comprehensive list of metabolites identified in this study and the extraction method most suitable for detection of each one. The acid−base methods due to salt production on neutralization are problematic for mass spectrometry applications but may be suited to other analytical platforms.

There are limitations to this investigation. For example, none of the methods were suitable for extraction of highly lipophilic metabolites, and it will be necessary to assess this in a separate study. Furthermore, GC/MS is not ideally suited to all classes of metabolites; for example, sugar phosphates are extremely labile,

making their detection difficult by GC/MS,[13,42,43] although we do illustrate detection in this investigation (Supporting Information Figure 1). However, the platform is regarded as the gold standard[44] and currently has the obvious advantage of having the most comprehensive databases for metabolite identification, thus allowing comparative monitoring of many areas of metabolism. It should be noted that no single analytical technique is applicable to the detection of *all* expected metabolites, but the robustness, accuracy, precision and wide metabolome coverage offered by GC/MS results in this analytical technique being one of the most suitable analytical platform presently available. NMR, CE/MS, and LC/MS platforms also have significant difficulties for metabolic profiling applications including sensitivity, detection efficiency, and accuracy (for example, matrix effects associated with electrospray ionization) and metabolite identification which requires two orthogonal properties (for example, retention time and fragmentation mass spectrum).

In conclusion, for anyone wishing to study intracellular metabolomics in *E. coli* the findings of this investigation indicate that the most suitable method to quench metabolism in *E. coli* is 60% methanol solutions (−48 °C). For the best coverage of the metabolites, we recommend pure methanol (−48 °C) with repeated freeze−thaw cycles as an extraction protocol, but global analysis will require use of complementary extraction methods to cover the entire metabolome. Finally, we advocate monitoring the footprint and supernatants following quenching so that appropriate calculation of intracellular metabolites is possible.

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## SUPPORTING INFORMATION AVAILABLE

Supporting Information Figure 1, heat map illustrating the relative ratio of the identified metabolites during the validation of quenching of metabolism; Supporting Information Table 1, identification of metabolites from the quenching supernatants and footprint samples illustrated in the Venn diagram (Figure 3); Supporting Information Table 2, identification of metabolites from the different extraction methods illustrated in the Venn diagram (Figure 5). This material is available free of charge via the Internet at http://pubs.acs.org.

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