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ORIGINAL ARTICLE

Evaluation of extraction processes for intracellular metabolite profiling of mammalian cells: matching extraction approaches to cell type and metabolite targets

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Abstract In this study we report on the optimisation of the technologies for generation of a global metabolomics profile for intracellular metabolites in Chinese hamster ovary (CHO) cells. We evaluated the effectiveness of a range of different extraction methods applied to CHO cells which had been quenched using a previously optimised approach. The extraction methods tested included cold methanol, hot ethanol, acid, alkali and methanol/chloroform plus combinations of these. The extraction of metabolites using two 100% methanol extractions followed by a final water extraction recovered the largest range of metabolites. For the majority of metabolites, extracts generated in this manner exhibited the greatest recovery with high reproducibility. Therefore, this was the best extraction method for attaining a global metabolic profile from a single sample. However, another parallel extraction method (e.g. alkali) may also be required to maximise the range of metabolites recovered (e.g. non-polar metabolites).

Keywords Metabolomics · Antibody · Glutamine synthetase · CHO · Bioprocessing · GC-MS

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Abbreviations

CHO	Chinese hamster ovary
AMBIC	Ammonium bicarbonate
PCA	Perchloric acid
GC-MS	Gas chromatography-mass spectrometry

1 Introduction

Metabolomics approaches have been used extensively for understanding/interpreting responses to environmental stimuli in yeast and bacteria (Allen et al. 2003; Bundy et al. 2007; Tweeddale et al. 1998, 1999; Chassagnole et al. 2002) and have also been applied in studies in plants and higher eukaryotic systems (Fiehn 2002; Sreekumar et al. 2009). The knowledge gained from metabolomics studies in bacteria, yeast and plants is now being applied in the area of biotechnological processes and is allowing rapid development of sampling and data analysis methods (Ma et al. 2009; Khoo and Al-Rubeai 2009; Sellick et al. 2009). However, the study of mammalian systems has predominantly focused on examination of metabolic profiling (biomarkers) from blood, serum and other body fluids (Sreekumar et al. 2009; Kenny et al. 2008; Heazell et al. 2008; Hollywood et al. 2006; Goodacre 2007). There are several compelling reasons for the application of metabolomics to mammalian cell culture systems for production of protein therapeutics, especially to provide information to underpin rational medium design and metabolic engineering (Park et al. 2005), but this area has only recently started to be a focus of interest. Reports have started to appear of metabolomic studies in mammalian cells including adherent cultured human fibroblasts (Bennett et al. 2008), human breast cancer cell lines (Teng et al. 2009), Madin–Darby

canine kidney (MDCK) (Ritter et al. 2008) and hepatic cells (Hofmann et al. 2008), as well as suspension cultured Chinese hamster ovary (CHO) and mouse NS0 myeloma cells (Sellick et al. 2009). Suspension cultured CHO and NS0 cells are particularly important for the biotechnology industry because the majority of commercially valuable protein biopharmaceuticals are produced using these cell types as "factories" for large-scale bioprocessing. This is due to the complex nature of these biopharmaceuticals that require appropriate folding and mammalian-type post-translational processing for full biological activity (Birch and Racher 2006; Butler 2005). The ability to apply a metabolomics approach to understand the interaction of cell culture environment with the efficiency of mammalian cell culture growth and biopharmaceutical production offers the potential to rationalise and optimise bioprocess design.

Current metabolite recovery protocols are generally two-step processes consisting of an initial quenching of the cells, to stop all metabolic activity and remove contaminating medium, followed by extraction of the metabolites. The methods for quenching and extraction are undoubtedly sample- and cell-dependent. Differences in cellular composition (especially cell membrane and cell wall structure) and cell size may influence the efficiency of quenching and the rate of metabolite leakage. Many metabolites are extremely labile, for example ATP and glucose 6-phosphate have turnover rates of less than 1–2 s (Weibel et al. 1974), and hence cellular metabolism must be stopped immediately (quenched) upon sampling of the cells to prevent/minimise metabolite turnover. We have previously characterised and optimised the quenching methods for the generation of physiologically relevant intracellular metabolite profiles from CHO cells and defined how the quenching process influences the recovery of a series of key intracellular metabolites (Sellick et al. 2009).

Following quenching, metabolites are extracted using methods that lyse the cells, allowing release of metabolites, ideally under conditions in which the majority of the metabolites are soluble, stable and in an enzymatically inert environment. Naturally, different extraction methods are biased towards different classes of compounds. Several different extraction methods have been reported including cold methanol (or methanol/water), hot ethanol or methanol, perchloric acid, potassium hydroxide and chloroform (or chloroform/methanol/water) extractions (Faijes et al. 2007; Maharjan and Ferenci 2003; Winder et al. 2008; Gonzalez et al. 1997; Hajjaj et al. 1998; Entian et al. 1977; Theobald et al. 1993; Chassagnole et al. 2002). Methods have also been developed for plant metabolomics that combine sequential solvent and water extractions (Mounet et al. 2007; Biais et al. 2009) or chloroform/methanol/water extractions where both polar and non-polar phases are used for analysis (Wu et al. 2008). In *E. coli*, cold methanol extraction is considered the best general method for metabolite profiling applications since it recovers the greatest range of metabolites (Winder et al. 2008; Maharjan and Ferenci 2003). However, different extraction methods have been recommended for mammalian cells ranging from 80% methanol for human fibroblast cells (Bennett et al. 2008) to boiling methanol or methanol/chloroform for MDCK cells (Ritter et al. 2008). To date, there have been no reports on the effectiveness of different metabolite extraction methods for CHO cells, which will be of great significance in defining optimal cell culture conditions for industrial scale production of biopharmaceuticals. In this paper, we report the effectiveness of a range of different extraction methods applied to CHO cells quenched with methanol and ammonium bicarbonate (AMBIC) at −40°C. We have also assessed the potential to combine a number of these extraction methods in an attempt to define the potential to use a single extraction procedure to extract the entire cellular complement of intracellular metabolites. We found that two 100% methanol extractions followed by one water extraction (defined as methanol + water) was the best overall method for metabolite extraction, since it combines the benefits of solvent extraction with those of water extraction. We also note that the recovery of metabolites from certain pathways require, or may be enhanced by, the use of alternative extraction procedures.

2 Materials and methods

2.1 Media and reagents

CD-CHO medium was obtained from Invitrogen (Carlsbad, US); the chemical composition is defined but confidential. All other reagents were obtained from Sigma-Aldrich unless otherwise stated.

2.2 Cell lines

The GS-CHO cell line LB01 was obtained from Lonza Biologics (Slough, UK), and was generated by transfection of CHOK1SV cells with a construct containing glutamine synthetase (GS) and a chimeric IgG4 antibody (cB72.3).

2.3 Cell culture and growth assessment

Stocks of LB01 cells were revived in 20 ml CD-CHO medium (Invitrogen), supplemented with 25 μM methionine sulfoximine (MSX). The cells were sub-cultured every 3–4 days with a seeding density of 0.2×10^6 cells ml^{-1} and were grown in 250 ml Erlenmeyer flasks in a volume of 50 ml medium. All cultures were grown at 37°C with

shaking at 100 rpm. Growth was assessed by light microscopy using an improved Neubauer haemocytometer at 24 h intervals. Samples were appropriately diluted and mixed 1:1 with 0.5% Trypan Blue in PBS.

2.4 Metabolite extraction

The cells were grown to mid-exponential phase (day 5) and then quenched before extraction. The cells were rapidly quenched by addition of 1×10^7 cells to 5 volumes of 60% methanol supplemented with 0.85% (w/v) AMBIC (pH 7.4) at −40°C. Addition of the cells to the quenching solution increased the temperature by no more than 15°C. The cells in the quenching solution were then centrifuged at 1,000×*g* for 1 min and the quenching solution was removed. The metabolites were extracted from the cells using a variety of methods as described below. The methods for the methanol, hot ethanol, alkali (potassium hydroxide; KOH), acid (perchloric acid; PCA) and methanol/chloroform extractions were adapted from Winder et al. (2008).

2.4.1 Methanol extraction

Three different methanol extractions were performed with different methanol concentrations (100, 80 and 60% in milliQ grade water). In each case, the cell pellet was resuspended in 0.5 ml of the appropriate methanol solution followed by flash freezing in liquid nitrogen. After thawing on ice at 4°C, samples were vortexed for 30 s, centrifuged at 800×*g* and the supernatant removed. The pellet was resuspended in a further 0.5 ml of the same methanol solution and the extraction repeated. The methanol extracts were pooled, centrifuged at 15,000×*g* for 1 min and the supernatant removed and lyophilised.

2.4.2 Methanol/water extraction

Two extractions with 100% methanol were performed, as described above, followed by an extraction in water. For the water extraction, the cell pellet was resuspended in 0.3 ml ice-cold milliQ water followed by flash freezing in liquid nitrogen. After thawing on ice at 4°C samples were vortexed for 30 s, centrifuged at 15,000×*g* and the supernatant removed. The methanol and water extracts were pooled, centrifuged at 15,000×*g* for 1 min and the supernatant removed and lyophilised.

2.4.3 Hot ethanol

The cell pellet was resuspended in 1 ml 90°C 100% ethanol and incubated at 90°C for 10 min. The samples were cooled on ice for 5 min, centrifuged at 15,000×*g* for 1 min and the supernatant removed and lyophilised.

2.4.4 Alkali (KOH) extraction

The cell pellet was resuspended in 1 ml 80°C 0.25 M KOH and incubated at 80°C for 10 min. The samples were cooled on ice for 5 min and neutralised with PCA. The precipitate was removed by centrifugation at 15,000×*g* for 5 min at 4°C and the supernatant removed and lyophilised.

2.4.5 Acid (PCA) extraction

The cell pellet was resuspended in 1 ml ice-cold 0.25 M PCA followed by flash freezing in liquid nitrogen. After thawing on ice at 4°C samples were vortexed for 30 s and flash frozen in liquid nitrogen. The freeze–thaw process was repeat twice more before the samples were neutralised with KOH. The precipitate was removed by centrifugation at 15,000×*g* for 5 min at 4°C and the supernatant removed and lyophilised.

2.4.6 Methanol/chloroform extraction

The cell pellet was resuspended in 1 ml methanol/chloroform solution (2:1) followed by flash freezing in liquid nitrogen. After thawing on ice at 4°C samples were vortexed for 30 s and flash frozen in liquid nitrogen. The freeze–thaw process was repeat twice more and either 0.4 ml ice-cold water or 0.5 mM tricine (pH 7.4) were added. The samples were vortexed for 30 s and centrifuged at 15,000×*g* for 1 min at 4°C to separate phases. The upper phase was removed and a further 0.25 ml ice-cold water or 0.5 mM tricine (pH 7.4) were added. The samples were vortexed for 30 s, centrifuged at 15,000×*g* for 1 min at 4°C to separate phases and the upper phase was removed. The upper phases were pooled and lyophilised.

2.4.7 Methanol/chloroform combined extraction

The extractions were performed as described for the methanol/chloroform extractions except that the lower chloroform phase was also removed and pooled with the upper phase before lyophilisation. This method was adapted from Wu et al. (2008).

2.4.8 Methanol/chloroform multi-step extraction

The cell pellet was resuspended in 1 ml methanol/chloroform solution (2:1) and flash frozen in liquid nitrogen. After thawing on ice at 4°C samples were vortexed for 30 s and flash frozen in liquid nitrogen. The freeze–thaw process was repeated twice more and 0.4 ml 0.5 mM tricine (pH 7.4) was added. The samples were vortexed for 30 s and centrifuged at 15,000×*g* for 1 min at 4°C to separate the phases and the upper phase was removed to a fresh

tube. Ice-cold 0.25 M KOH (0.25 ml) was added and the samples were vortexed for 30 s, centrifuged at 15,000×*g* for 1 min at 4°C to separate the phases and the upper phase was removed to a fresh tube. Ice-cold 0.25 M PCA (0.25 ml) was added, vortexed, centrifuged and the upper phase removed and pooled with the KOH fraction. The precipitate was removed by centrifugation at 15,000×*g* for 5 min at 4°C, the supernatant was removed and lyophilised. The lower chloroform phase was removed and pooled with the tricine fraction, transferred to the tube containing the lyophilised KOH/PCA fraction and lyophilised to create one sample.

2.5 NAD^+/NADH assays

The concentrations of NAD^+ and NADH were measured using an NAD^+/NADH Quantification Kit (BioVision) in 96 well plates. The method is described in Sellick et al. (2009).

2.6 Sample derivatization

Lyophilised intracellular metabolite extracts were prepared for GC-MS analysis by a two-stage derivatisation procedure. Metabolite pellets were resuspended in methoxyamine hydrochloride in pyridine (40 mg/ml; 10 µl) and incubated at 30°C for 90 min with gentle shaking. *N*-methyl-*N*-trimethylsilyltrifluoroacetamide with 1% trimethylchorosilane (MSTFA + 1% TMCS) (90 µl) was then added and incubated at 37°C for 30 min. The samples were cooled to room temperature and transferred into silanized GC vials (National Scientific) for GC-MS analysis.

2.7 GC-MS analysis

GC-MS analysis was performed on a 7890A GC System (Agilent Technologies) coupled to a 5975C Inert XL MSD with Triple-Axis Detector (Agilent Technologies) using the manufacturers software (MSD ChemStation). Samples (1 µl with 10:1 split ratio) were injected onto a DB-5MS + DG column (Agilent Technologies; 250 µm × 30 m, 0.25 µm film thickness with 10 m duraguard) using helium (1.2 ml/min) as the carrier gas. Components were separated by isothermal chromatography for 1 min at 60°C, followed by an increase to 325°C at a rate of 10°C/min then 10 min at 325°C. Mass spectra were acquired in positive ion mode using electron impact ionisation at 70 eV. The injector, MS source and MS quad temperatures were set at 250, 230 and 150°C, respectively. Mass spectra were scanned from 50 to 600 mass units. Metabolite peaks in the raw chromatograms were identified using MSD ChemStation (Agilent Technologies) to search the Agilent Fiehn GC/MS Metabolomics RTL Library (http://fiehnlab.ucdavis.edu/Metabolite-Library-2007), and automated mass spectral deconvolution and identification system software (AMDIS) (National Institute of Standards and Technology (NIST); http://www.amdis.net) to search the NIST/EPA/NIH Mass Spectral Library (http://www.nist.gov/srd/nist1a.htm). Metabolite identifications were based on retention times and fragmentation patterns. The data were combined using an in-house Microsoft Excel (Microsoft Corporation) macro and normalized to the retention time locking (RTL) compound (myristic acid d_{27}).

3 Results and discussion

3.1 Efficiency of extraction methods

In an effort to define the most efficient extraction method for global metabolite profiling, eight different extraction methods were assessed following quenching of the CHO cells in 60% methanol + 0.85% AMBIC at −40°C. The methods tested were extraction with 100, 80 or 60% methanol, 100% methanol followed by a water extraction, hot ethanol, alkali (KOH), acid (PCA) and methanol/chloroform (see Sect. 2 for details). To assess the efficiency of the extractions, and hence the recovery of metabolites from the quenched cell pellet, the samples were analysed by GC-MS. Comparison of the raw chromatograms showed significant differences in the number and amounts of metabolites extracted under each condition (Fig. 1). The greatest number of metabolite peaks was obtained from extractions using either the cold solvent-based methods (100, 80 and 60% methanol or 100% methanol + water) or methanol/chloroform. These methods each generated approximately 210 individual peaks, with the methanol + water method extracting the greatest number (Fig. 2). The KOH extraction had ~25% fewer (152 peaks) and PCA had even fewer (~70%, 59 peaks). Approximately a third of the total peaks detected in each extraction condition (except PCA) could be putatively identified [According to MSI standards (Sumner et al. 2007)] as corresponding to specific metabolites (Fig. 2). In the case of PCA extractions, >50% of the peaks corresponded to metabolites and this increased identification rate may result from the PCA extraction recovering only the most stable metabolites that are also most amenable to GC-MS analysis. However, this advantage must be set against the major disadvantage that very few peaks could be detected. A complete list of all identified metabolites for each extraction process is detailed in Fig. 3.

It was important to determine whether the metabolite features observed in the different extracts represented the same or different metabolites. As expected, the

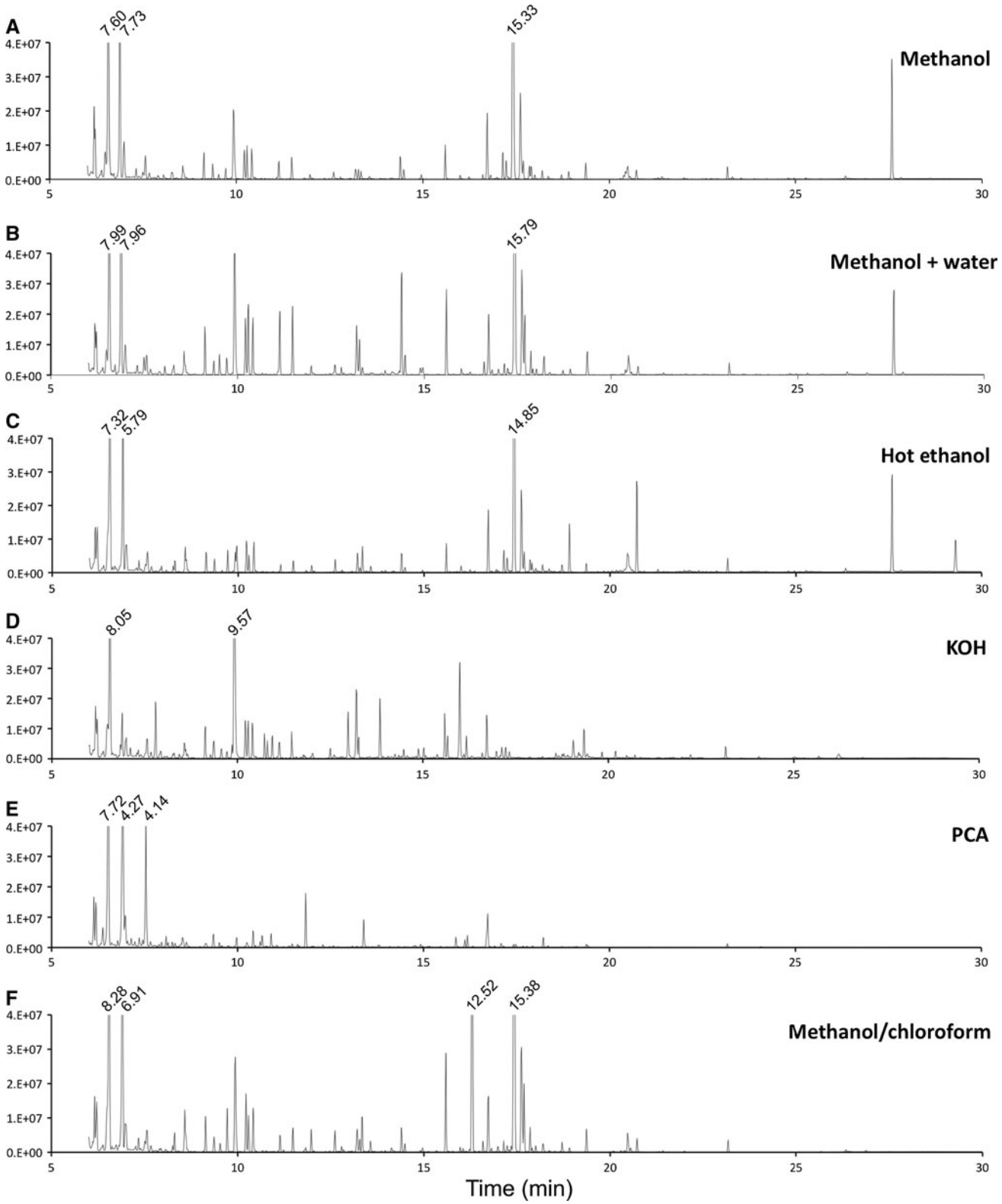

Fig. 1 Raw GC-MS traces for different extraction methods. Intracellular metabolites quenched with methanol + AMBIC were extracted with either **a** methanol, **b** methanol + water, **c** hot ethanol, **d** KOH, **e** PCA or **f** methanol/chloroform. The *y*-axis has been manually set to 4×10^7 ion counts to highlight the differences between extraction methods

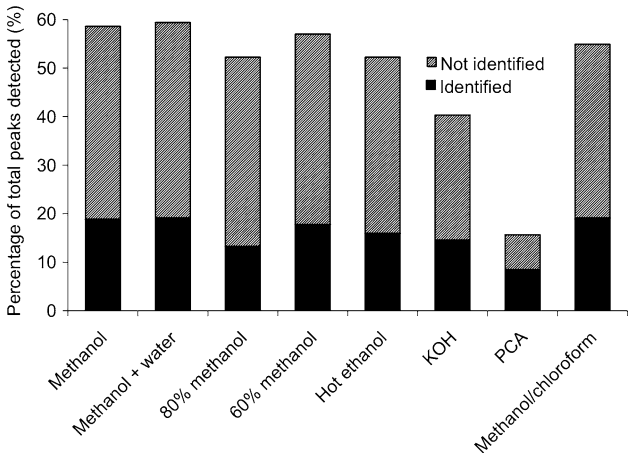

Fig. 2 The range of metabolites recovered and detected by each extraction method. Peaks identified for each extraction method are presented as a percentage of the total number of unique peaks detected across all extraction methods. The numbers of peaks assigned to specific metabolites are shown in *black*. Metabolites were identified by screening of the detected peaks against the current Agilent Fiehn GC/MS Metabolomics RTL and NIST/EPA/NIH Mass Spectral metabolite libraries

methanol-based extractions recovered a large number of the same metabolites with 147 out of 224 metabolites observed in all the extractions and a further 68 present in at least two of the three methods (Fig. 4a). In contrast, comparison of methanol + water extractions with KOH and PCA extractions showed that of all the peaks only 11 were shared by the three conditions and only 50% of the KOH peaks and 33% of the PCA peaks were observed in the methanol + water extractions (Fig. 4b). This means that, of a total of 341 unique peaks identified in the three extractions, over 75% of the peaks were only observed in one of the three extractions. Although the acidic or alkaline conditions may extract different classes of metabolites, it is also possible that these conditions cause chemical modifications that could result in non-physiological profiles. Finally, comparison of methanol + water, hot ethanol and methanol/chloroform extractions showed 117 peaks were present in all three extractions. However 57 peaks were unique to methanol + water with 34 and 38 unique peaks present in hot ethanol and methanol/chloroform, respectively (Fig. 4c).

The use of heat in some of the extraction methods (hot ethanol and KOH) may affect the range of metabolites recovered due to the loss of heat labile metabolites and a build-up of degradation products. However, hot ethanol extraction did not seem to adversely affect the number or range of metabolites detected using GC-MS (Figs. 2, 4c). To demonstrate the negative effects of extractions using heat, the levels of the labile metabolite NAD^+, and the more stable NADH, were determined (Fig. 5). The NAD^+ recovery clearly showed that whilst 100% methanol recovered relatively large amounts of NAD^+, the use of heat in the hot ethanol extractions, and the combination of

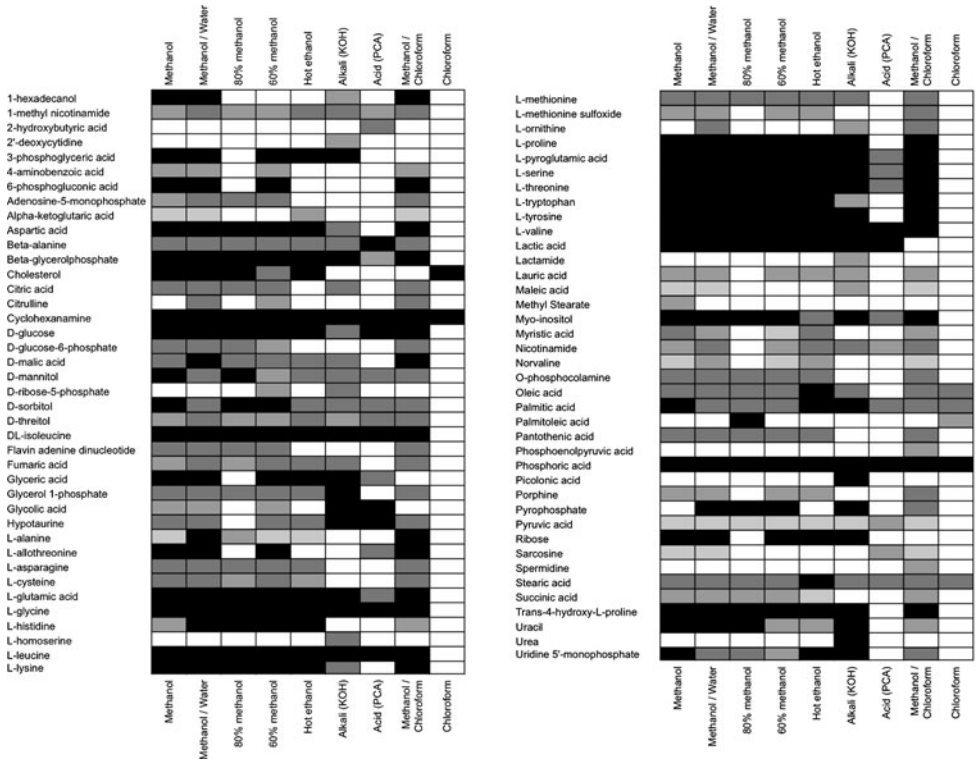

Fig. 3 A heatmap detailing the relative amounts of the identified metabolites recovered by each of the different extraction methods. Each value represents the average of four independent samples normalised to an internal standard. Response ratios above 1 are *coloured black* and below 0.0001 are *coloured white*. The remaining values have been split into four groups (1–0.1, 0.099–0.01, 0.0099–0.001, 0.00099–0.0001) and coloured in decreasing greyscale

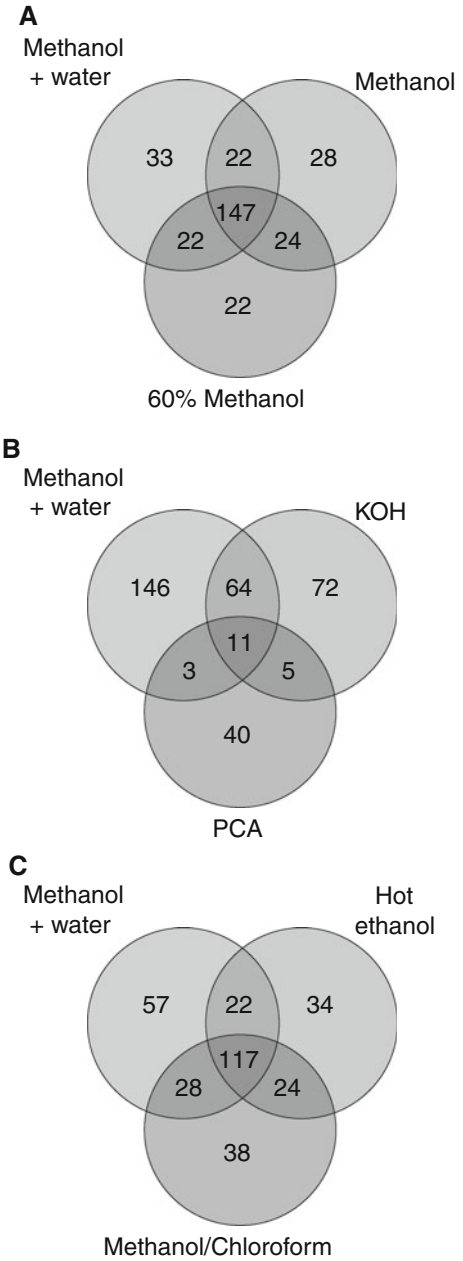

Fig. 4 *Venn diagrams* highlighting the common GC-MS peaks observed with each extraction method. In each case the methanol + water extractions have been used as the benchmark. Peaks are counted if they appear in all four of the replicates

heat and alkaline conditions in the hot KOH extractions, resulted in decomposition of almost all of the NAD^+. Whilst PCA extraction was also detrimental due to the acid hydrolysis of the ribosyl pyridinium bond in NAD^+. Changing the methanol:water ratio of the extraction solution modified the recovery of NAD^+ and NADH. When the ratio was lower, a decreased recovery of NAD^+ and increased recovery of NADH was obtained. This finding suggests that methanol concentrations of 100% are needed for optimal recovery.

3.2 Defining optimal extraction processes for metabolite profiling

Having shown that the profile of metabolites that can be recovered from suspension CHO cells is dependent on the extraction method used, we elected to examine the influence of extraction method on the recovery of metabolites that can be used to build models of cellular metabolism. Accurate physiological profiling of core metabolites is critical for meaningful modelling of the interaction between cells and their environment and for generation of hypotheses that link environment to the efficiency of recombinant protein generation. To profile the extraction efficiency, we examined the recovery of 24 metabolites, including 12 amino acids (Figs. 6, 7).

A clear trend was observed with the amino acids, where methanol + water and methanol/chloroform extraction methods yielded the greatest amounts of almost all of the amino acids (Fig. 6). Furthermore, similar amounts of each amino acid were recovered with each of these extractions. Although there were some exceptions (aspartate, asparagine, leucine and methionine), in general, the amino acids assessed were more soluble in water-based extractions (methanol + water and methanol/chloroform methods) than with other methanol-based methods. The increased water content in the 60% methanol extraction did not prove to be a significant advantage over 100% methanol for any of these amino acids.

KOH extraction generally proved to be as effective as 100% methanol (but not as effective as the combination of methanol + water) in terms of amino acid recovery, but there were differences in relation to selective recovery of specific amino acids (Fig. 6). For example, with KOH there was poor recovery of asparagine, leucine, lysine, tyrosine and tryptophan. Acid extraction presented a similar limited profile of recovery. Generally, the recovery of amino acids with PCA extraction was very low which suggests that amino acids may show limited solubility under these conditions of extraction.

Analysis of metabolites from a range of different pathways to determine metabolic class extraction efficiency (Fig. 7) showed that, once again, methanol + water and methanol/chloroform extractions were generally most effective, in terms of spectrum of recovery and the amount recovered of the majority of each of the 12 metabolites. Cholesterol was a notable exception and was only recovered in solvent-based extraction methods. Decreasing methanol:water ratios were paralleled by decreased cholesterol recovery (Fig. 7a). PCA extraction, although generally poor in terms of spectrum and amount of recovery, resulted in preferential recovery of threitol (Fig. 7b). Some compounds were only extracted by methods containing at least 40% water without extremes

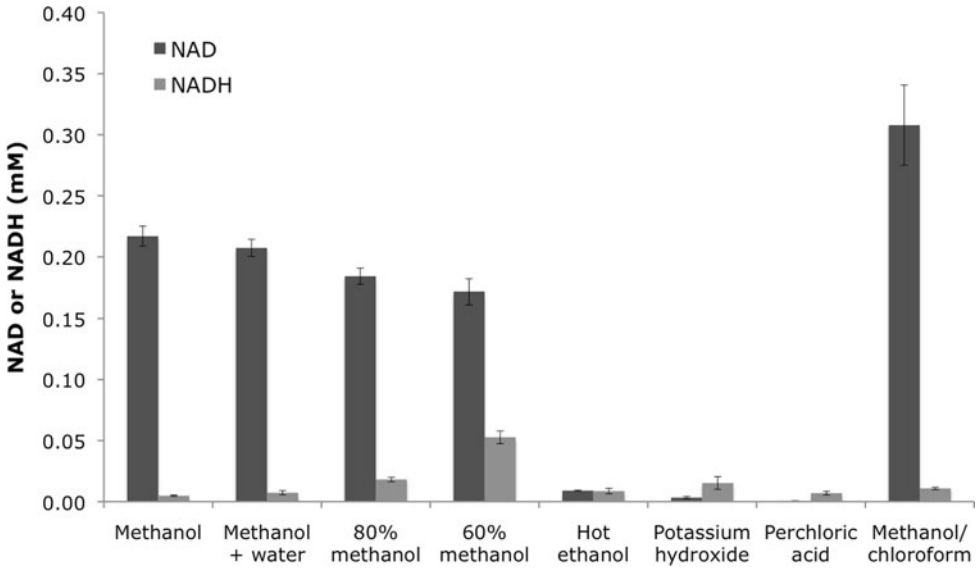

Fig. 5 Recovery of NAD^+ and NADH from quenched cell pellets using different extraction methods. Values for each experiment represent the average of three biological replicates with two technical replicate readings of each, and standard deviation *error bars* are shown

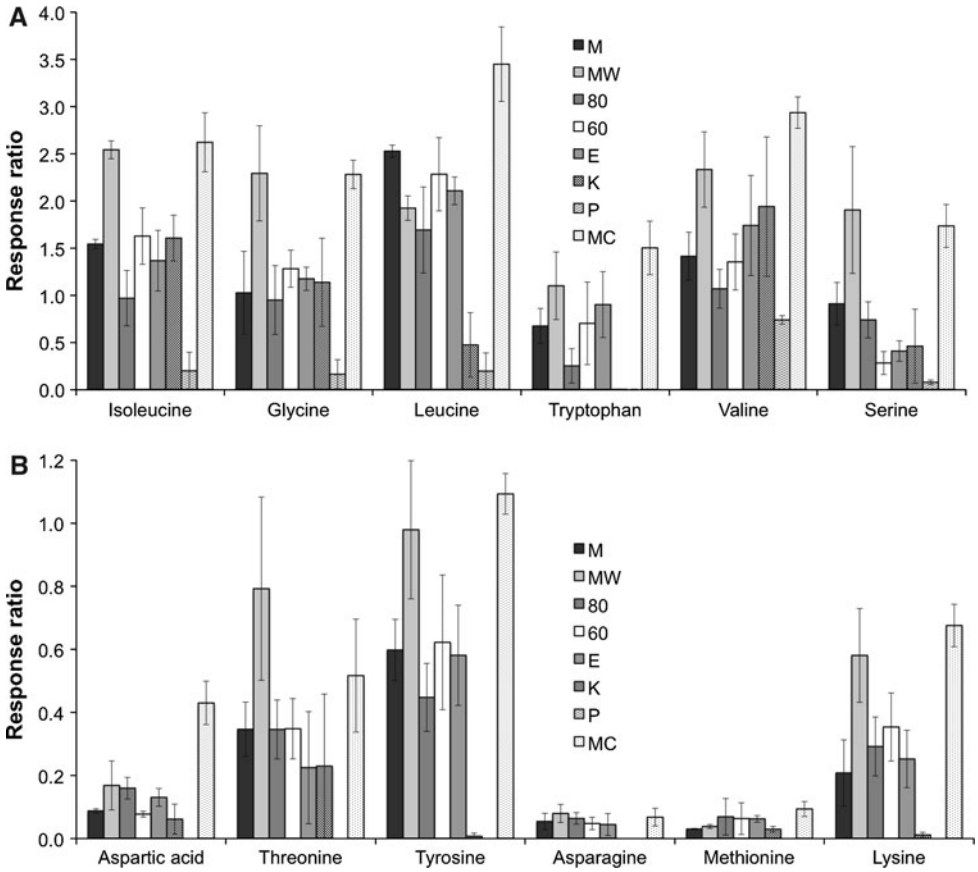

Fig. 6 Twelve of the amino acids identified in the different extraction methods. Each *bar* represents the average of four independent extractions normalised to an internal standard. The amino acids have been divided into those with a response ratio above or below 1.5 (**a** and **b**, respectively). *Error bars* represent standard deviations

of pH. For example, citrulline was not recovered in solvent-based extractions or acid or alkali extractions (Fig. 7b). These data demonstrate that the addition of a water-based extraction step to the 100% methanol extractions increased the amount and range of metabolites recovered compared to solvent alone. This combination, as illustrated by others (Mounet et al. 2007; Biais et al. 2009), combines the advantages of both methanol- and water-based methods. However, using 60% or 80% methanol did not, in general, improve recovery of metabolites, and often resulted in lower recovery of metabolites since these extractions were not suitable for

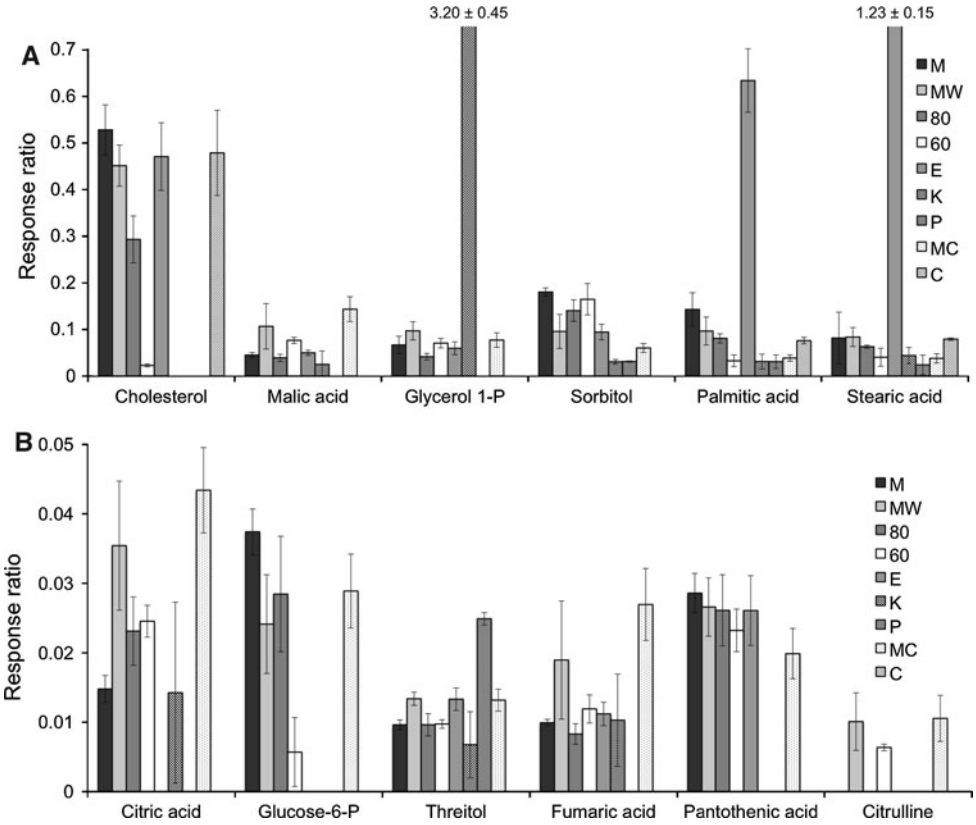

Fig. 7 Twelve key metabolites identified in the different extraction methods. Each *bar* represents the average of four independent extractions normalised to an internal standard. The key metabolites have been divided into those with a response ratio above or below 0.1 (**a** and **b**, respectively). *Error bars* represent standard deviations

metabolites that solely required either solvent or aqueous conditions (e.g. cholesterol, fatty acids and citrulline).

Although extractions with KOH had been shown to generate a limited profile of intracellular metabolites, glycerol-1-phosphate recovery was significantly greater when cellular metabolites were extracted with KOH (about 30-fold greater recovery than observed for any other procedure) (Fig. 7a). This may reflect saponification of phospholipids and/or triglycerides by KOH, due to hydrolysis of the ester links. This would release potassium salts of the fatty acids together with glycerol-1-phosphate. However, KOH extraction did not increase the recovery of fatty acids (i.e. oleic acid, palmitic acid and stearic acid). In fact, a decrease is observed compared to the best methods for extracting fatty acids.

Extraction with hot ethanol provided excellent recovery of fatty acids (e.g. stearic acid and palmitic acid). It is likely that this was due to the combination of heat and organic solvent increasing the solubility of the fatty acids. As with cholesterol detection (see above), decreasing the methanol:water ratio significantly decreased the recovery of fatty acids (Fig. 7a). These data highlight the importance of using appropriate extraction processes for targeted analysis of specific metabolic pathway components.

Our analyses with different extraction methods have shown that each provides advantages and disadvantages for extraction of metabolites, depending on their physical properties. Therefore, the overall value of intracellular metabolite profiling may be limited by method selection. An ideal extraction method would enable the recovery of the broadest range of metabolites. Towards this end, we hypothesised that a combination of extraction processes (sequentially or with pooling of extracts) would increase the number and extent of recovery of the metabolites. We assessed the validity of this hypothesis in two combination protocols based around methanol/chloroform extractions (as detailed in Sect. 2). In the first combination protocol we pooled the upper (methanol/tricine) and lower (chloroform) phases of the methanol/chloroform extraction, since the latter should add metabolites such as cholesterol and fatty acids, which are extracted preferentially into the chloroform phase. The second combination protocol used KOH and subsequent PCA extractions, following removal of the methanol/tricine phase from the methanol/chloroform extraction. In both protocols, combining extracts improved the range of metabolites extracted when compared to the individual extraction methods (data not shown). However sample-to-sample variability increased dramatically due to the increased number of extraction steps. Coefficients of variance across all identified metabolites increased from $\sim$20% in single extractions to >100% in the combined extractions (data not shown). The advantages in terms of

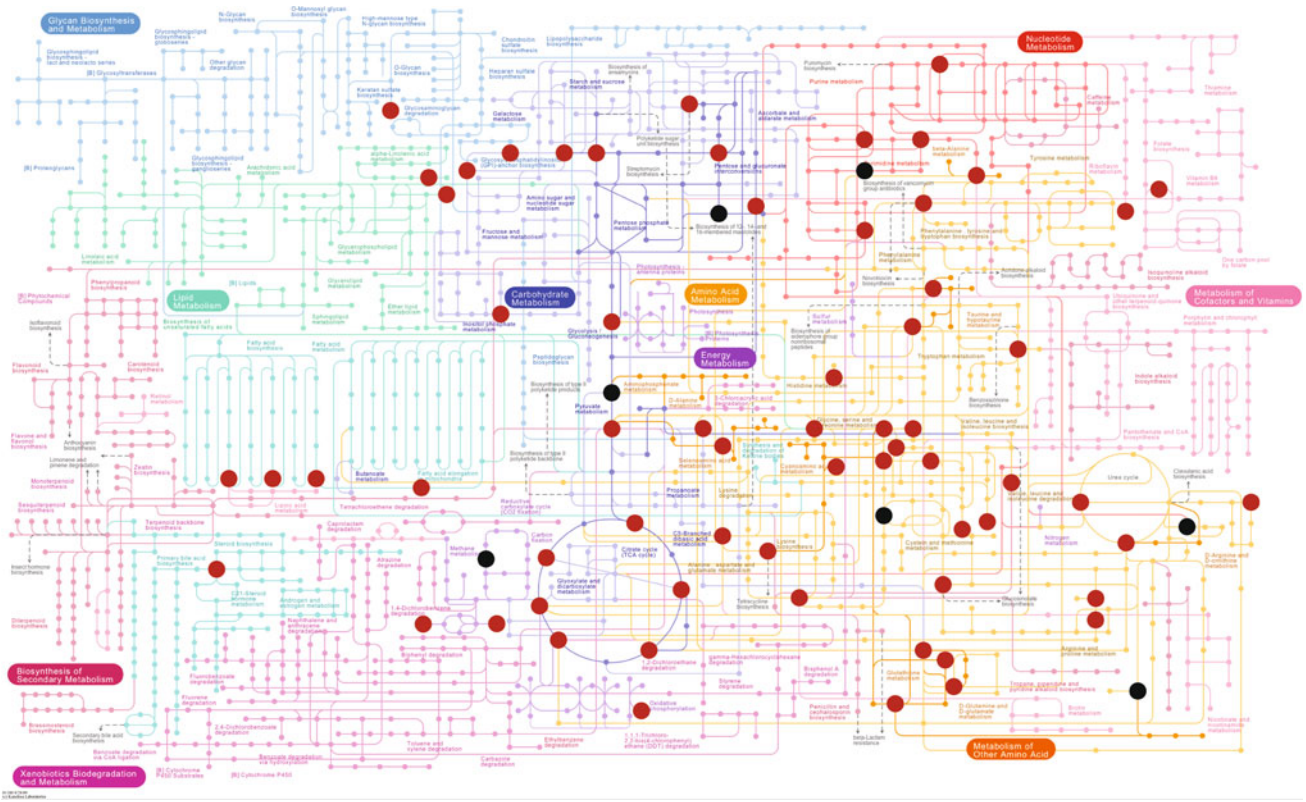

Fig. 8 The range of metabolites extracted using the extraction protocols. The metabolites that have been extracted and positively identified have been mapped onto the KEGG metabolite pathway map to highlight the coverage achieved. The metabolites recovered using the methanol + water extraction method are indicated by light [red] spots and those requiring isolation by other methods are indicated by dark [black] spots. KEGG metabolite pathway map taken from http://www.genome.jp/kegg/kegg1.html (Color figure online)

enhanced metabolite profiling by combination protocols were largely outweighed by the decreased reproducibility and accuracy in recovery and, in their current format, the combination extraction protocols were unsuitable for routine determination of intracellular metabolites.

Overall, we would recommend the use of the methanol + water extraction method as the best general method. It combines the best properties of both the solvent- and water-based methods and, within a single approach, recovers the greatest range of metabolites (Fig. 3). In most cases, extracts generated in this manner exhibit the greatest amounts of recovered metabolites. The methanol + water extractions were also the most reproducible (RSD = 18% compared to 19% for methanol/chloroform, 22% for 100% methanol and 24% for hot ethanol for the 24 metabolites shown in Figs. 6 and 7). Mapping the profile of metabolites recovered from methanol + water extractions onto metabolic pathway maps demonstrates that this method provides coverage of key metabolites from all the main metabolic pathways and regulatory points in cellular metabolism (Fig. 8; light [red] spots). Increased coverage can be achieved by using more than one extraction method (Fig. 8; dark [black] spots). Based on these data, our optimised method for extraction of intracellular metabolites from suspension cultured CHO cells, and other similar cell types (e.g. mouse NS0 myeloma cells), consists of quenching the cells in 60% methanol + 0.85% AMBIC followed by extraction of the metabolites from the cells using two 100% methanol extractions followed by a water extraction.

4 Conclusions

We have now fully defined a standard protocol for quenching (Sellick et al. 2009) and extraction of metabolites from CHO cells. The extraction of physiologically valid levels of metabolites is critical for the modelling of metabolic processes and this study defines the best conditions for the extraction and analysis of 89 unique metabolites using GC-MS. Whilst methanol + water extraction results in recovery of the greatest range of metabolites, no method provides the optimal conditions for extraction of all metabolites. Therefore, additional, complementary extraction methods should be used in parallel when broader metabolite profiles are required, or for targeted analysis of

non-polar metabolites. The application of metabolite profiling to CHO cell metabolism under defined culture conditions offers great potential for enhancement of industrial cell line selection and process development and hence overall yield and profitability.

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