Application of a differential method for reliable metabolome analysis in chemostat cultivated *Escherichia coli*

Hilal Taymaz-Nikerel[1], *Marjan de Mey*[2], *Cor Ras*[1], *Angela ten Pierick*[1], *Reza M. Seifar*[1], *Jan C. van Dam*[1], *Joseph J. Heijnen*[1], *Walter M. van Gulik*[1]

[1]Department of Biotechnology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands; telephone: +31-15-2784629; fax: +31-15-2782355

e-mail: w.m.vangulik@tudelft.nl

[2]Laboratory of Industrial Microbiology and Biocatalysis, Department of Biochemical and Microbial Technology, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium

Abstract

Quantitative metabolomics of microbial cultures requires well designed sampling and quenching procedures to obtain proper snapshots of intracellular metabolite levels. In the present work we successfully applied a differential method to obtain a reliable set of metabolome data for *Escherichia coli* K12 MG1655 grown in steady state, aerobic, glucose limited chemostat cultures. From a rigorous analysis of the commonly applied quenching procedure based on cold aqueous methanol, whereby metabolite analyses were carried out in total broth, culture filtrate, cell pellets and the obtained supernatants after quenching and washing of the cell pellets, it was concluded that cold methanol quenching was not applicable because it resulted in release of a major part of the metabolites from the cells. No positive effect of buffering (with HEPES or tricine) or increasing of the ionic strength of the quenching solution by addition of NaCl was observed. Application of a differential method, whereby metabolite measurements in total broth samples are corrected for the metabolites present in the culture supernatant, in principle requires metabolite measurements in two samples, that is of total broth and filtrate, for each measurement. Different methods for sampling of culture filtrate were examined and it was found that direct filtration without cooling of the sample prior to filtration was the most appropriate procedure. Analysis of culture filtrates revealed that most of the central metabolites and amino acids are present in significant amounts outside the cells in chemostat cultivated *E. coli* K12 MG1655 cells. Due to the fact that the turnover time of the pools of extracellular metabolites, which is in chemostat cultures equal to the reciprocal of the dilution rate, is much larger than the turnover time of the intracellular pools the differential method should also be applicable to short term pulse response experiments without requiring measurement of metabolites in the supernatant during the dynamic period.

Introduction

Metabolic engineering of cells requires comprehensive knowledge and understanding of the functionality and control of the metabolic reaction network as a whole. Hence, *in vivo* kinetic studies are vital in facilitating understanding, manipulating and modeling of metabolic reaction networks.

In vivo kinetic studies rely partly on rapid dynamic perturbation experiments during which a steady-state chemostat culture is perturbed and the subsequent response in both extracellular and intracellular metabolites is monitored (Theobald *et al*., 1997; Chassagnole *et al*., 2002; Visser *et al*., 2002; Mashego *et al*., 2004; Wu *et al*., 2005). Taking into account the high turnover rate of most intracellular metabolites, proper measurement of their concentrations requires rapid broth sampling and instantaneous quenching of enzyme activity. The significance of correct measurement of intracellular concentrations of metabolites is obvious from the fact that wrong determinations will lead to unrealistic conclusions on the status of the metabolism and hence on the kinetics of the enzymes.

There have been several attempts to develop reliable sampling methods for quantification of intracellular metabolites in bacteria. Table 1 presents the different published sampling, quenching and extraction methods which have until now been applied for metabolite analysis in chemostat grown *Escherichia coli* K12 cells.

Rapid sampling of cells from a bioreactor with the aim to capture highly dynamic changes of intracellular metabolites in pulse response studies has been achieved by developed fast techniques comprising immediate withdrawal of culture broth into syringes or tubes containing a certain quenching fluid. Theobald *et al*., 1993 developed a fast manual sampling technique with which samples could be withdrawn in a fraction of a second with intervals of 2 - 5 seconds between samples. A comparable system has been developed by Lange *et al*., (2001). To achieve shorter intervals between samples Schaefer *et al*., (1999) constructed an automated sampling device with a maximum sampling frequency of 4.5 samples.s^{-1}. Another sampling device, which

66 was based on the stopped-flow technique, was introduced by Buziol *et al*., 2002 with which
67 dynamic responses in the millisecond range could be captured. Recently Schaub *et al*., 2006
68 proposed an integrated sampling procedure; whereby the sampling is carried out through a
69 helical single tube heat exchanger.
70 The most commonly used method for fast quenching of all metabolic activity in culture samples
71 has been direct sampling into cold aqueous 60% (v/v) methanol at -40^{o}C. This method was
72 firstly applied for yeast (de Koning and van Dam, 1992). Generally removal of the extracellular
73 metabolites present in the broth is performed by centrifugation of the quenched broth/quenching
74 liquid solution to obtain a cell pellet. Centrifugation of the broth/quenching liquid mixture is
75 done at temperatures below -20^{o}C (Schaefer *et al*., 1999). Liquid nitrogen was also used for
76 quenching of *Escherichia coli* (Chassagnole *et al*., 2002), as well as the above mentioned rapid
77 heating of the broth by means of a heat exchanger (Schaub *et al*., 2006). The disadvantage of
78 these methods is that removal of extracellular metabolites is not possible.
79 Removal of extracellular metabolites is a pre-requisite for proper quantification of the
80 intracellular metabolite levels. It should be realized here that although the extracellular
81 concentrations of most metabolic intermediates are generally much lower than the intracellular
82 concentrations, the amounts present in the supernatant may still be very significant because the
83 volume of the supernatant in laboratory cultivations is typically two orders of magnitude larger
84 than the total cell volume. It has been reported that using the currently most used quenching fluid
85 aqueous methanol (Table 1) for bacteria results in loss of intracellular metabolites due to leakage
86 to the quenching liquid (Wittmann *et al*., 2004; Bolten *et al*., 2007). Bolten *et al*., compared the
87 cold methanol quenching method with separation and washing of the cells by means of filtration
88 for different gram-positive and gram-negative bacteria including *Escherichia coli* K12 (DSMZ
89 2670) grown in shake-flasks. Depending on the micro-organisms and the metabolites, cold
90 methanol quenching either resulted in higher or lower measured intracellular concentrations
91 compared to filtration. Because the filtration procedure took about 30 seconds per sample, this

method appears not to be suitable for rapid sampling in dynamic experiments and neither for steady state measurements of metabolites with short turnover times. Even for yeast cells which are thought to be more robust, leakage of metabolites during quenching has been observed to occur (Villas-Boas *et al*., 2005). Villas-Boas and Bruheim (2007) suggested a quenching method whereby cold glycerol-saline is used as the quenching solution. They compared it with the conventional aqueous methanol solution and showed that the metabolite levels were much higher when samples were quenched with the cold glycerol-saline solution. However, as they also mentioned in their work, the application of glycerol leads to additional problems because it is difficult to remove from the samples

Subsequently the quenched cells have to be extracted to release the intracellular metabolites. For *Escherichia coli* perchloric acid extraction has been the most widely used procedure (Schaefer *et al*., 1999; Buchholz 2001, 2002; Chassagnole *et al*., 2002). In some cases different methods have been used for the extraction of different classes of metabolites, for example alkaline extraction for NADH and NADPH (Chassagnole *et al*., 2002). Maharjan and Ferenci (2003) investigated the influence of different extraction methods i.e. hot ethanol, cold and hot methanol, perchloric acid, alkaline and methanol-chloroform, on the quantification of metabolites extracted from *E. coli*. They suggested using of cold methanol because of its simplicity and the fact that it allowed more components to be obtained. Hiller *et al*., 2007 claimed that usage of buffered hot water (30 mM TEA, pH 7.5, 95 °C) results in more reliable metabolite extraction compared to buffered ethanol, unbuffered hot water or perchloric acid for *E. coli*.

For the quantification of the metabolites in the cell extracts different kinds of analytical techniques have been applied, such as enzymatic assays, thin-layer chromatography, gas chromatography (GC), nuclear magnetic resonance (NMR) and high-performance liquid chromatography (HPLC). Presently mass spectrometry (MS) has become more popular as the detection method, thereby coupled to GC, capillary electrophoresis (CE) or HPLC as initial separation step. More advanced LC-MS/MS methods allow more accurate, robust and sensitive

118 analyses (van Dam *et al*., 2002; Luo *et al*., 2007). For application of LC-MS/MS especially the
119 rigorous use of stable isotope (e.g. ^{13}C) labeled internal standards is a prerequisite to obtain
120 reproducible and reliable results (Mashego *et al*., 2004; Wu *et al*., 2005).
121 The aim of the present study was to obtain a fast and reliable method for sampling and
122 quenching of *E. coli* K12 cells, which would also be applicable to highly dynamic pulse response
123 studies, carried out in chemostat cultures. Having shown that considerable amounts of
124 metabolites are present outside the cells, different variations of a quenching protocol based on
125 cold aqueous methanol were investigated. A rigorous approach was used to quantify metabolite
126 leakage. This approach was based on demonstration that decrease in amounts of metabolites
127 present in the cell pellets is quantitatively matched with a corresponding increase in metabolites
128 in the quenching and washing fluids. Glycolytic, pentose phosphate pathway and TCA cycle
129 intermediates as well as amino acids and adenyl-nucleotides were measured in order to quantify
130 leakage of these compounds into the quenching and washing fluids. As alternative we applied a
131 differential method, whereby from the quantification of metabolites in both the broth and the
132 supernatant a reliable metabolite dataset was obtained for steady state cultivated *E. coli* in
133 aerobic glucose limited chemostat culture.
134

135 ## Materials and Methods

136 **Strain and preculture conditions**

137 The *Escherichia coli* K12 MG1655 [λ^-, F^-] strain was obtained from The Netherlands Culture
138 Collection of Bacteria (NCCB). Cells were grown to stationary phase in shake-flasks on LB
139 medium. Culture aliquots containing 50% (v/v) glycerol were kept at -80^{o}C until they were used
140 as inoculum of the precultures for chemostat experiments.
141 Precultures were grown on minimal medium with the following composition per liter: 5.0 g
142 $(NH_4)_2SO_4$, 2.0 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g NaCl, 2.0 g NH_4Cl, 0.55 g glucose.$1H_2O$,
143 0.001 g thiamine-HCl, 1 mL of trace elements solution (Verduyn *et al*., 1992) and 40 mM

MOPS. The pH of the medium was adjusted to 7.0 with 1M K_2HPO_4 before filter sterilization (pore size 0.2µm, cellulose acetate, Whatman GmbH, Germany).

Chemostat cultivation

Aerobic glucose-limited chemostat cultures were carried out on minimal medium at a dilution rate (D) of 0.1 h^{-1} either in 7 L laboratory fermentors with a working volume of 4 L, controlled by weight, or in 1 L laboratory fermentors with a working volume of 0.5L, controlled by means of an overflow system, (Applikon, Schiedam, The Netherlands). The composition of the minimal medium was, per liter: 1.25 g $(NH_4)_2SO_4$, 1.15 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g NaCl, 6.75 g NH_4Cl, 30 g glucose.$1H_2O$, 0.001 g thiamine-HCl, 2 mL of trace elements solution (Verduyn *et al*., 1992) and 0.2 mL silicone based antifoaming agent (BDH, Poole, UK). This medium allowed a steady state biomass concentration under glucose limited conditions of about 11 gDW/L. The medium was filter sterilized (pore size 0.2µm, polyethersulfone, Sartorius, Goettingen, Germany) without pH adjustment, the final pH of the medium being 4.90. The cultivation temperature was controlled at 37^{o}C, and the pH was controlled at 7.0 with 4M KOH. The 7 L fermentor was operated at 0.3 bar overpressure, a stirrer speed of 500 rpm (two 6-bladed Rushton turbine stirrers, D=85mm) and an aeration rate of 100L/h. The 1 L fermentor was operated at 0.1 bar overpressure, a stirrer speed of 700 rpm (two 6-bladed Rushton turbine stirrers, D=45mm) and an aeration rate of 30L/h. Under these conditions oxygen transfer was sufficient because the dissolved oxygen tension (DOT) never dropped below 50% of air saturation which was measured online with a DOT sensor (Mettler-Toledo GmbH, Switzerland). During all chemostat experiments the oxygen and carbon dioxide concentrations in the offgas, DOT, pH, temperature, reactor vessel weight (only for 7 the L fermentor), effluent vessel weight and the added amounts of base+antifoam were monitored online. The carbon dioxide and oxygen volume fractions in dried (permapure, Perma Pure LLC, USA) offgas were monitored online

with a combined carbon dioxide (infrared) and oxygen (paramagnetic) gas analyzer (NGA 2000, Fisher-Rosemount, Germany).

Medium feeding was started when the carbon dioxide evolution rate (CER) and the oxygen uptake rate (OUR) during the batch phase preceding the chemostat cultivation, which was carried out on a medium identical to the feed medium, declined to nearly zero. The chemostat was assumed to be in steady-state after five residence times which was verified from the measured biomass concentration and the online measurements of dissolved oxygen and the oxygen and carbon dioxide concentrations in the offgas.

Measurement of cell dry weight

From a broth sample 4x10g was transferred to centrifuge tubes, the cells were spun down (at 5000g, 4^{o}C, for 5 min) and were washed twice with 0.9% NaCl solution. The centrifuge tubes containing the cell pellets were dried in an oven at 70 °C for 48 hours until constant weight. The cell dry weight was obtained gravimetrically.

Determination of residual glucose and total organic carbon

Samples of the supernatant were obtained by rapid sampling of broth into syringes containing cold stainless steel beads followed by immediate filtration, according to the procedure described by Mashego *et al*. (2003). The glucose concentration in these samples was analyzed enzymatically (Boehringer Mannheim/R-Biopharm, Roche). The total amount of organic carbon (TOC) in the broth and filtrate were quantified with a TOC Analyzer (TOC-5050A, Shimadzu).

Rapid sampling

Broth sampling: Using a home built rapid sampling system (Lange *et al*., 2001), which was coupled to the fermentor, samples of 1 mL broth were withdrawn from the fermentor within less than one second. Samples were withdrawn directly into tubes containing 5 mL of quenching

195 solution precooled at -40 °C, which were immediately mixed after sampling by vortexing. The
196 exact sample sizes were quantified gravimetrically by weighing the tubes before and after
197 sampling.
198
199 *Filtrate sampling:* Samples of extracellular culture fluid were obtained with three different
200 methods: (1) syringe filtration with cold stainless steel beads (Mashego *et al*., 2003), (2) syringe
201 filtration at room temperature without beads, (3) centrifugation at 5000g, 4^{o}C, for 5 min. After
202 removal of the cells with one of these three methods, the obtained filtrate or supernatant was
203 immediately mixed with 5 mL of quenching solution in order to process these samples in the
204 same way as the broth samples. Also in this case the exact amount of sample obtained was
205 quantified gravimetrically.
206
207 **Quenching procedure**
208 The quenching solutions that were used were: 1. 60% (v/v) aqueous methanol, 2. 60% (v/v)
209 aqueous methanol + 70mM HEPES, 3. 60% (v/v) aqueous methanol + 0.9% NaCl, 4. 60% (v/v)
210 aqueous methanol + 10mM Tricine. After quenching of broth samples in one of these quenching
211 solutions, precooled at -40^{o}C, the sample/quenching solution mixture was centrifuged for 5 min
212 at 8000g in a cooled centrifuge (-20 °C) using a rotor which was precooled at -40 °C. After
213 decanting, the supernatant (QS) was stored at -40 °C until extraction. Subsequently the cell
214 pellets were resuspended in 5 mL of -40 °C quenching solution and again centrifuged. Also this
215 second supernatant (WS) was stored at -40 °C until extraction. For measurement of metabolites
216 in total broth as well as in the culture filtrate the same quenching procedure was applied,
217 however, the quenched total broth mixtures (B) or quenched culture filtrates (F) were not
218 centrifuged but after thorough vortexing 500 µL of these mixtures were withdrawn for metabolite
219 extraction.
220

Metabolite extraction procedure

Extraction of metabolites from the cell pellets as well as from the 500 µL samples from the quenched total broth was performed with the hot ethanol method (Gonzalez *et al*., 1997). In order to analyze the metabolites in the supernatants obtained from the quenching and washing procedure (QS and WS) and quenched culture filtrate (F), 500 µL of each of these solutions were subjected to the same procedure as for cell pellets. In all cases 100 µL of U-^{13}C internal standard mixture was added to the pellets and samples before extraction. Metabolites were extracted in 75% boiling ethanol (3 min, 90 ^{o}C). After cooling the thus obtained extracts were evaporated to dryness in a RapidVap (Labconco Corporation, Kansas, Missouri, USA) during 110 min under vacuum. After resuspension of each residue in 500 µL of H_2O, cell debris was removed by centrifugation during 5 min at 5000g. After decanting the supernatants were stored at -80^{o}C until further analysis.

The reason that in all cases (cell pellets, filtrates and supernatants) the same quenching and extraction procedure was applied was to obtain analytes with as much as possible the same sample matrix. In addition the boiling ethanol step assured the destruction of any enzymatic activity which could cause conversion of metabolites before of during the analysis procedure.

Metabolite analysis

Metabolites of the glycolysis, TCA cycle and PPP were quantified with Isotope Dilution Mass Spectrometry (Wu *et al*., 2005). Amino acids were quantified with GC-MS, using the kit EZ:Faast® Free (Physiological) (Phenomenex, Torrance, CA, USA). The concentrations of the adenine nucleotides AMP, ADP and ATP were also analyzed with IDMS, the applied LC-ESI-MS/MS procedure has been described in Wu *et al*. (2006).

Flux Balance Analysis (FBA)

A minimal reaction set capable of describing the growth of *E. coli* in aerobic glucose limited chemostats was derived from the genome scale model of Reed *et al*., 2003. Based on this minimal set, consisting of 275 reactions, a determined stoichiometric model was constructed. This model was applied to carry out flux balance analysis, whereby the measured rates of glucose uptake, oxygen consumption, biomass growth, carbon dioxide production and byproduct formation during steady state chemostat growth were used as input variables. The ATP balance was not used as constraint in the flux balancing procedure.

Results and Discussion

Chemostat cultivations

The aim of this work was to develop a reliable sampling method for quantification of intracellular metabolites in *E. coli* applicable to steady state chemostat cultures as well as to highly dynamic pulse response experiments. In this study all cultivations were aerobic glucose limited chemostats on a minimal medium, carried out at a dilution rate of 0.1 h^{-1}. For all chemostat cultivations measurements were performed during steady state growth of residual glucose, biomass concentration, oxygen and carbon dioxide concentrations in the offgas and medium, inflow and culture outflow rates. In addition to this the TOC concentration of the culture filtrate was measured in order to verify if significant amounts of byproducts were formed. From these measurements, the biomass specific rates of glucose consumption, biomass growth, oxygen consumption and carbon dioxide production were calculated (see Table 2). Chemostat cultivations were carried out in two different bioreactors, with working volumes of 4 and 0.5 L. All chemostat experiments appeared to be well reproducible and no significant differences were observed between the 4 and the 0.5 L. cultivations, with respect to the biomass specific conversion rates, as can be inferred from the average rates calculated for the three 4 L and the two 0.5 L chemostat cultivations which were carried out (see Table 2).

271 Although the growth conditions were aerobic and carbon limited it appeared from TOC
272 measurements that the culture filtrate still contained a significant amount of organic carbon
273 (about 20% of the TOC in the broth). It was verified from the residual glucose measurement and
274 HPLC analysis of the filtrate, however, that concentrations of glucose and organic acids were
275 negligible (results not shown). Therefore the measured residual organic carbon was attributed to
276 cell lysis. An indication for this was that the measured carbon to nitrogen (C/N) ratio of the
277 filtrate was very similar to the C/N ratio of the biomass (results not shown). If all organic carbon
278 in the filtrate is considered as lysed biomass, the calculated specific rate of biomass decay was
279 considerable (approx. $0.02h^{-1}$), which implies that the specific growth rate, μ, in the chemostat,
280 being the sum of the dilution rate and the rate of cell decay, was higher than the dilution rate,
281 namely $\mu \sim 0.12h^{-1}$.
282 The measured biomass yield in our chemostat cultivations was Y_{SX}=3.2 CmolX/mol glucose
283 which is very similar to published biomass yields of this *E. coli* strain under similar conditions
284 (Pramanik and Keasling, 1997; Nanchen *et al*., 2006). From the last two columns of Table 2 it
285 can be seen that the recoveries of carbon and redox were close to 100% for both the 4 L and the
286 0.5 L chemostats.
287
288 **Separation of cells and supernatant**
289 It has been shown that in bacterial cultures significant amounts of several central metabolites and
290 amino acids are present in the culture filtrate (Bolten *et al*., 2007). These authors also showed
291 that the applied filtration procedure could result in additional leakage of metabolites from the
292 cells. In order to ensure accurate quantification of metabolites in the culture supernatant, three
293 different separation methods for obtaining the filtrate were compared, namely (F_1) quenching of
294 the broth sample with cold stainless steel beads and subsequent filtration (Mashego *et al*., 2003),
295 (F_2) direct filtration of the broth sample without cooling and (F_3) direct centrifugation of the
296 broth (see Materials and Methods). Figure 1 presents the results of the measurements of a

297 number of different central metabolites and amino acids in the broth (B) and in the filtrate for the
298 three different filtrate sampling techniques (F_1, F_2, F_3). The data in Figure 1 are averages from
299 four replicate samples taken from two independent chemostat experiments. From these
300 measurements it can be inferred that the amount of metabolites measured in filtrate depend on
301 the separation method applied to obtain filtrate. It was observed that method F_1 (filtration after
302 cooling the broth with cold beads to a temperature close to 0 ^{o}C) resulted in almost all cases in
303 the highest amounts of measured metabolites in the filtrate. For the cases where indeed
304 significant differences were found, direct filtration (F_2) resulted in the lowest amounts of
305 measured metabolites. Apparently rapid cooling of the broth and, to a lesser extent
306 centrifugation, resulted in release of part of the metabolites from the cells. The observation that
307 rapid cooling of the broth resulted in release of metabolites from the cells has been observed
308 before in *Corynebacterium glutamicum* and has been attributed to a high sensitivity to cold
309 shock (Wittmann *et al*., 2004). According to these results direct filtration of the broth without
310 cooling was chosen as the preferred method to obtain culture supernatant for metabolite
311 quantification.
312
313 **Metabolite measurements in broth and supernatant**
314 In order to quantify whether central metabolites of *E. coli* are also present in significant amounts
315 in the culture filtrate during steady state chemostat cultivation, metabolite measurements were
316 carried out in the broth as well as in the filtrate of steady state chemostat cultures. The measured
317 concentrations of metabolites in the filtrate, the amounts of metabolites in the broth and in the
318 filtrate (both expressed per g dry weight of biomass), and the corresponding percentages of the
319 total amounts of metabolites which are present in the filtrate are shown in Table 3. From these
320 results it can be inferred that most of the measured metabolites are present in significant
321 quantities, if expressed per amount of biomass, in the culture filtrate.

322 The fraction of the metabolite outside the cells appeared to vary between the different
323 metabolites. However, no clear trends can be observed. One of the reasons that most of the
324 measured metabolites are present in the culture supernatant is probably cell lysis. As has been
325 argued above, the measured TOC content and C/N ratio of the filtrate indicate that the biomass
326 decay rate might have been as high as 1/6 of the growth rate of the culture. If the release of
327 metabolites to the supernatant would have been caused by cell destruction alone, and no re-
328 consumption by the living cells would have occurred, the fraction of metabolites in the
329 supernatant would have been 1/6 of the total for each metabolite. The fact that for more than half
330 of the measured metabolites this fraction is significantly higher than 1/6 indicates that there must
331 exist other processes which cause release of metabolites from the cells.
332 So far most authors considered the presence of metabolites of glycolysis, TCA cycle or PPP in
333 the supernatant negligible, because the levels were below the detection limit of their analysis
334 methods (Schaub *et al*, 2006 and Hiller *et al*, 2007) However, this clearly does not agree with the
335 present findings. Due to the sensitivity of the applied LC-ESI-ID-MS/MS analysis method
336 metabolite concentrations well below 1 µM can be quantified. Due to the fact that the volume of
337 the supernatant is two orders of magnitude larger than the total cell volume, these low
338 concentrations represent a significant amount of metabolites. Table 3 shows that 0.55 µM
339 isoleucine still contributes to more than 30% of the total isoleucine present. In correspondence
340 with our findings also Bolten *et al*. (2007) detected intermediates from the glycolysis, TCA cycle
341 and PPP in the supernatant for some bacterial species.
342 From the above it is clear that total broth extraction, as used in several published studies
343 (Chassagnole *et al*., 2002, Schaub *et al*., 2006) cannot be used for proper measurement of
344 intracellular metabolite levels. Therefore the metabolites present outside the cells have to be
345 eliminated prior to the metabolite extraction procedure. The usual approach, to avoid the
346 interference of metabolites in the filtrate, is to use the cold methanol quenching method
347 combined with cold centrifugation, with or without a washing step. The advantage of this

method is that metabolism is instantaneously stopped and that all compounds which are present outside the cells can be effectively removed. However, according to several studies quenching with cold aqueous methanol prior to separation might lead to additional leakage of intracellular metabolites from the cells, especially in case of bacteria. To determine losses of metabolites from the cells when the cold methanol quenching method is applied we used a rigorous balancing approach, whereby the metabolites in all different fractions (broth, filtrate, cell pellet, quenching and washing solutions) were quantified.

Validation of the cold methanol quenching method for chemostat cultivated *E. coli*

Steady state broth samples were rapidly withdrawn from the chemostat with a dedicated rapid sampling system and directly quenched in 60% methanol solution at -40 ^{o}C according to Lange *et al*. (2001). In order to quantify metabolite leakage metabolite measurements were performed in the broth (B), culture filtrate (F), quenching solution (QS), washing solution (WS) and in the cell pellets (IC). This allowed us to verify, from the mass balance for each metabolite, if metabolite losses could indeed be attributed to leakage because the measured amounts in the different fractions should add up to the amount measured in the broth. The results for a number of representative metabolites from the glycolysis, TCA cycle and PP-pathway are shown in Figure 2 and represent averages of four replicate samples taken from two independent steady state chemostat experiments. It can be seen from this Figure that the mass balances close well in most cases (>95%). From the metabolite measurements in broth and filtrate the intracellular amount could be calculated (IC_{cal} in the left columns). For all metabolites the measured amounts in the cell pellets (IC) were much lower than the calculated amounts (IC_{cal}) indicating that the major part of the intracellular metabolites ended up in the quenching (QS) and washing (WS) solutions. This clearly shows that quenching of *E. coli* cells in cold methanol results in release of the major part of the intracellular metabolites. Subsequently it was investigated if buffering of the 60% cold methanol or the addition of salt could reduce the leakage of metabolites during

374 quenching. Therefore quenching of the samples was also carried out with a.) 60% (v/v) aqueous
375 methanol + 70mM HEPES, b.) 60% (v/v) aqueous methanol + 0.9% NaCl, and c.) 60% (v/v)
376 aqueous methanol + 10mM Tricine, all kept at -40^{o}C. However, no significantly different results
377 were obtained for these three alternatives compared to the 60% cold methanol without any
378 additions (not shown).
379 As alternative for the cold methanol quenching procedure Villas-Boas and Bruheim (2007)
380 applied cold glycerol-saline as quenching solution for *S. cerevisiae* and *Streptomyces coelicolor*.
381 Although the measured metabolite amounts in the cell pellets were consistently higher than if
382 cold methanol quenching was applied, they could not show if this method completely avoided
383 leakage of metabolites, because no metabolite measurements were carried out in the broth and in
384 the filtrate. A disadvantage of applying mixtures containing glycerol as quenching liquid is the
385 fact that it cannot be evaporated and thus cannot be completely removed from the cell pellet.
386 Until now no proper alternative for the cold methanol quenching and washing procedure has
387 been proposed which was demonstrated not to result in leakage of intracellular metabolites. An
388 alternative method for the determination of intracellular metabolites in *E. coli*, whereby
389 metabolite leakage can be circumvented, is the differential method, proposed but not applied by
390 (Mashego *et al*., 2007; Bolten *et al*., 2007; Schaub *et al*., 2006) which was in fact applied above
391 to determine the intracellular metabolite amounts from the metabolite measurements in broth and
392 culture filtrate (IC_{cal}).
393
394 **Application of the differential method**
395 The differential method was applied for quantification of central metabolites and amino acids in
396 aerobic glucose limited chemostat cultures run at dilution rate of 0.1 h^{-1}. After steady state was
397 reached broth samples were rapidly withdrawn from the chemostats and directly quenched and
398 filtrate samples were obtained by direct filtration (see materials and methods). After extraction,
399 metabolites were analyzed in the broth and in the filtrate. The results are shown in Table 3. Two

400 different chemostat systems were used, with working volumes of 4 and 0.5 L, and the results of
401 the metabolite measurements appeared to be very similar for both systems. The averages of
402 intracellular metabolite measurements from at least three independent runs are presented in the
403 last column of Table 3, which also includes the intracellular ATP, ADP and AMP levels.
404 Remarkably, these metabolites could not be detected in the filtrate, as they were below the
405 detection limit of the analysis procedure.
406 A possible drawback of the proposed differential method would be that in case of high
407 metabolite concentrations in the filtrate two relatively large numbers are subtracted from each
408 other resulting in large errors in the calculated intracellular amounts. It can be observed from the
409 results in Table 3 that the metabolite with the highest fraction present in the filtrate was 6PG
410 (80%), all others were much lower (50% or less). However, the reproducibility of the data
411 obtained from replicate chemostat cultivations resulted in relatively low standard errors (Figure 2
412 and Table 3) therefore making the method also applicable to the metabolites that occur in
413 significant amounts outside the cells.
414 An important aspect for meaningful metabolome quantification is that the sampling and
415 quenching of the cells is sufficiently fast to obtain a proper snapshot of the actual metabolite
416 concentrations. Thereby the time between the withdrawal of the sample and the complete arrest
417 of all metabolic activity by the quenching procedure, should be significantly shorter than the
418 turnover times of the intracellular metabolite pools. The residence time of the cells in the applied
419 rapid sampling device, that is the traveling time from the chemostat to the tube containing the
420 quenching solution, was below 100 milliseconds, whereas the mixing time of the broth with the
421 quenching solution was estimated to be less than 80 milliseconds (Lange *et al*., 2001 and
422 references therein). The turnover times of the intracellular metabolite pools can be estimated
423 from the calculated metabolic fluxes for the applied cultivation conditions and the measured pool
424 sizes of the metabolites. Using a stoichiometric metabolic model derived from the genome scale
425 metabolic model for *E. coli* K12 MG1655 of Reed *et al*., (2003) metabolic flux balancing was

applied whereby the measured macroscopic conversion rates during steady state aerobic glucose limited chemostat cultivation were used as inputs. Subsequently the turnover times of the intracellular metabolites were calculated from the measured intracellular concentrations (see Table 3) and the total conversion rates obtained from the flux balancing with the stoichiometric model. The obtained results for a number of representative metabolites are shown in Table 4. It can be seen from this table that especially the turnover times of the intermediates of the central metabolic pathways are in the seconds to sub-seconds range. It can be concluded from these results that the applied sampling procedure should be fast enough to obtain proper measurements of the intracellular levels of free amino acids as well as the majority of the central metabolites.

Quality check of the obtained metabolome data

Although the intracellular metabolite levels of *E. coli* K12 during glucose limited steady state chemostat cultivation that have been published so far are in most cases of the same order of magnitude, there are big variations. These variations might have been caused by the different sampling/quenching techniques and/or the different analysis procedures applied. Either total broth extractions have been performed or cells have been quenched in cold methanol resulting in leakage. Furthermore the limit of detection of the applied analysis methods may have varied significantly and measurements in the noise level range should be considered with care. The application of ^{13}C internal standards has considerably increased the accuracy of the results. Taking this into account, the metabolite data published so far should be interpreted with caution, as they might not represent the real situation.

However, without ending up in complicated thermodynamic considerations (Kümmel *et al*., 2006) a few checks can be carried out to examine the quality of metabolome data. First of all it is well known that for healthy growing cells the adenylate energy charge (ATP+ADP/2)/(ATP+ADP+AMP) is generally stable at values around 0.8 to 0.9 (Chapman *et al*., 1971). As has been shown by Bolten *et al*., (2007) for many published metabolite datasets of

452 *E. coli* the calculated e-charge appears to be far outside this range, which is indicative for
453 erroneous measurements. From our data an energy charge of 0.78 can be calculated, which is in
454 the expected range.
455 The quality of the metabolome data can further be checked by calculating the mass action ratios
456 (MAR) for reactions which are known to operate close to equilibrium. For these reactions the
457 calculated MAR should be close to the equilibrium constant (K_{eq}). Using our metabolite data the
458 MARs of some known near-equilibrium reactions were calculated and compared with the
459 published equilibrium constants (see Table 5). It can be seen from this table that the MAR of the
460 reactions catalyzed by phosphoglucose isomerase (PGI), mannose-6-phosphate isomerase (PMI),
461 adenylate kinase (AK), fumarase (FUM), and phosphoglyceromutase (PGM) + enolase (ENO)
462 are all very close to K_{eq}. This is a good indication of the thermodynamic feasibility of the data
463 gathered by the differential method.
464 The results of measurements of metabolites participating in NAD^+ dependent
465 oxidation/reduction reactions operating close to equilibrium can also be used to estimate the *in-*
466 *vivo* NAD^+/NADH ratio, which is an important parameter in physiological studies. By assuming
467 that the enzyme mannitol-1-phosphate dehydrogenase operates near to equilibrium and assuming
468 an intracellular pH of 7.0 the NAD^+/NADH ratio was calculated from the measured F6P and
469 Mannitol-1-P levels to be equal to 48. By assuming that the enzymes malate dehydrogenase and
470 aspartate transaminase operate close to equilibrium an NAD^+/NADH ratio of 71 was calculated
471 (details of the calculations are shown in the Appendix). Remarkably the estimated cytosolic
472 NAD^+/NADH ratio is very close to the value found for *Saccharomyces cerevisiae* under similar
473 conditions (Canelas *et al*., 2008).
474
475 **Can the differential method be applied to dynamic experiments?**
476 In order to capture the highly dynamic changes in metabolite levels during pulse response
477 experiments, the sample frequency should be sufficiently high. Taking into account the required

478 sample frequency in such experiments carried out with *E. coli*, which is in the order of seconds
479 to sub-seconds (Schaefer *et al.*, 1999; Buziol *et al.*, 2002), the withdrawal of two samples for
480 each single measurement is clearly not feasible. The question is, however, how fast the expected
481 changes are in the levels of the metabolites which are present in the culture supernatant. It should
482 be realized here that during steady chemostat cultivation the turnover time of all extracellular
483 metabolites is equal to the liquid residence time, which is equal to the working volume of the
484 reactor divided by the feed rate and thus equal to the reciprocal of the dilution rate. In case of a
485 chemostat with a dilution rate $D = 0.1\ h^{-1}$ the liquid residence time, and thus the turnover time of
486 the pool of extracellular metabolites is 10 hours. This implies that within the time span of a pulse
487 response experiment, which is in the order of seconds to minutes, no significant changes are
488 expected to occur if the rate of release of these metabolites remains at steady state level during
489 the pulse response experiment. If this is the case measurement of the steady state levels of the
490 metabolites of interest in the culture supernatant would be sufficient, which is something which
491 has to verified beforehand. Therefore the application of the differential method for dynamic
492 measurements will most probably require only broth sampling.
493

494 ## Conclusions

495 In this work it has been shown from highly sensitive LC-ESI-ID-MS/MS measurements of
496 metabolite levels in the culture supernatant of aerobic glucose-limited chemostat cultivated *E.*
497 *coli* cells that although the absolute concentrations are low, significant total amounts of
498 metabolites are present outside the cells due to the large supernatant volume. This implies that
499 total broth extraction cannot be carried out to obtain meaningful intracellular metabolite levels
500 and that effective removal of extracellular metabolites is necessary prior to metabolite extraction.
501 The most commonly applied method to remove extracellular compounds is cold methanol
502 quenching and subsequent centrifugation. From our thorough investigation on the fate of central
503 metabolites and amino acids upon quenching of *E. coli* cells with this method it can be

concluded that significant leakage of intracellular metabolites into the cold aqueous methanol solution occurs. Therefore this method cannot be applied. Because appropriate alternative methods to remove the extracellular metabolites before extraction are not available, the only way to obtain reliable results is to apply a differential method, whereby measurements of the metabolites in the total broth are corrected with measurement of the metabolites in the culture supernatant. Due to the large turnover time of the metabolites present in the culture supernatant in case of chemostat cultivation, this method is also applicable to short term pulse response experiments, where metabolite measurements of total broth extracts can be corrected with steady state measurements in the culture filtrate. In order to be able to apply the differential method the method for the quantification of metabolites should be highly sensitive and reproducible. The LC-ESI-ID-MS/MS method we used for metabolite analysis has proven to be a powerful tool that minimizes variations in analysis as a result of partial loss or degradation during the sample processing procedure, the analysis or instrument drift. Figure 3 summarizes the proposed procedure as a result from this work. The application of this procedure to steady state chemostat cultures yielded a reliable metabolome dataset for *E. coli* K12 MG1655 under these conditions. We believe that further application of this method will yield more appropriate data of levels of intracellular metabolites that will offer more realistic integration of metabolome data with other omics data into large scale mathematical models.

Appendix

The NAD^+/NADH ratio was calculated in two different ways under the assumption that reactions displayed in the table below operate close to equilibrium.

	Reaction	K_{eq}		Reference
1	Mannitol-1P + NAD^+ ⇌ F6P+NADH+H^+	K_1	$7.9*10^{-3}$ (pH=7.0)	Kiser and Niehaus,1981
2	malate + NAD^+ ⇌ oxaloacetate+NADH+H^+	K_2	$2.33*10^{-5}$ (pH=7.0)	Wilcock and Goldberg, 1972
	aspartate+αKG ⇌ oxaloacetate + glutamate	K_3	0.145	Kishore et al., 1998

The in-vivo NAD^+/NADH ratios were calculated as follows:

1 $$\frac{NAD^+}{NADH} = \frac{F6P}{Mannitol\text{-}1P}\frac{1}{K_1} = 48.4$$

2 $$\frac{NAD^+}{NADH} = \frac{Asp\ \alpha KG\ K_3}{Glu\ Mal\ K_2} = 71.3$$

Abbreviations

ADP	Adenosine-5-diphosphate
AK	Adenylate kinase
Ala	Alanine
AMP	Adenosine-5-monophosphate
Asp	Aspartate
ATP	Adenosine-5-triiphosphate
B	Broth
cGMP	Cyclic guanosine monophosphate
ENO	Enolase
F	Filtrate
FBP	D-Fructose 1,6-bisphosphate
FUM	Fumarase
F2,6bP	D-Fructose 2,6-bisphosphate
F6P	D-Fructose 6-phosphate
Glu	Glutamate
Gly	Glycine
G3P	Glycerol 3-phosphate
G6P	D-Glucose 6-phosphate
IC	Intracellular
K	Equilibrium constant of the enzyme
Mal	Malate
M6P	D-Mannose 6-phosphate
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide reduced
PEP	Phosphoenolpyruvate
PGI	Phosphoglucose isomerase
PGM	Phosphoglyceromutase
PGP	3-Phospho-D-glyceroyl phosphate
PMI	mannose-6-phosphate isomerase
T6P	Trehalose 6-phosphate
2PG	D-Glycerate 2-phosphate
3PG	3-Phospho-D-glycerate
6PG	6-phospho-D-gluconate
α KG	α-ketoglutarate

Acknowledgements:

The authors wish to thank the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT Vlaanderen) for financial support via the MEMORE project (174WT105).

References

Bolten, C. J., Kiefer P., Letisse F., Portais J.C., Wittmann C., 2007, 'Sampling for Metabolome Analysis of Microorganisms', Analytical Chemistry 79, 3843-3849.

Buchholz, A., Hurlebaus J., Wandrey C., Takors R., 2002, 'Metabolomics: quantification of intracellular metabolite dynamics', Biomolecular Engineering 19, 5-15.

Buchholz, A., Takors R., Wandrey C., 2001, 'Quantification of Intracellular Metabolites in *Escherichia coli* K12 Using Liquid Chromatographic-Electrospray Ionization Tandem Mass Spectrometric Techniques ', Analytical Biochemistry 295, 129-137.

Buziol, S., Bashir I., Baumeister A., Claaßen W., Noisommit-Rizzi N., Mailinger W., Reuss M., 2002, 'New Bioreactor-Coupled Rapid Stopped-Flow Sampling Technique for Measurements of Metabolite Dynamics on a Subsecond Time Scale', Biotechnol Bioeng 80, 632-636.

Canelas A.B., van Gulik W.M., Heijnen J.J., 2008, 'Determination of the cytosolic free NAD/NADH ratio in *Saccharomyces cerevisiae* under steady-state and highly dynamic conditions. Biotechnol Bioeng. (Epub ahead of print).

Chassagnole, C., Noisommit-Rizzi N., Schmid J.W., Mauch K., Reuss M., 2002, 'Dynamic Modeling of Central Carbon Metabolism of *Escherichia coli*', Biotechnol Bioeng 79, 53-73.

Chapman, A.G., Fall, L., Atkinson, D.E., 1971, Adenylate energy charge in *Escherichia coli* during growth and starvation. J. Bacteriol. 108:1072-1086.

de Koning, W., van Dam K., 1992, 'A Method for the determination of Changes of Gtycolytic Metabolites in Yeast on a Subsecond Time Scale Using Extraction at Neutral pH', Analytical Biochemistry 204, 118-123.

Gonzalez, B., Francois J., Renaud M., 1997, 'A Rapid and Reliable Method for Metabolite Extraction in Yeast using Boiling Buffered Ethanol', Yeast 13, 1347-1356.

Grisolia, S., Carreras J., 1975, 'Phosphoglycerate mutase from yeast, chicken breast muscle, and kidney (2,3-PGA-dependent)', Methods Enzymol. 42, 435-450.

Hiller J., Franco-Lara E., Weuster-Botz D., 2007, 'Metabolic profiling of *Escherichia coli* cultivations: evaluation of extraction and metabolite analysis procedures', Biotechnol. Lett. 29, 1169-1178.

Keruchenko, J.S., Keruchenko I.D., Gladilin K.L., Zaitsev V.N., Chirgadze N.Y., 1992, 'Purification, characterization and preliminary X-ray study of fumarase from *Saccharomyces cerevisiae*', *Biochimica et Biophysica Acta* 1122, 85-92.

Kiser, R.C., Niehaus W.G., 1981, 'Purification and Kinetic Characterization of Mannitol- 1 – Phosphate Dehydrogenase from Aspergillus niger', Archives of Biochemistry and Biophysics 211 (2), 613-621.

Kishore, N,Tewari YB, Goldberg RN, 1998, 'An investigation of the equilibrium of the reaction{L-aspartate(aq)+2-oxoglutarate(aq)=oxaloacetate(aq)+L-glutamate(aq)}', J. Chem. Thermodynamics 30, 1373-1384.

Kümmel A., Panke S., Heinemann M., 2006, Systematic assignment of thermodynamic constraints in metabolic network models. BMC Bioinformatics. 23(7);512.

Lange, HC, Eman M., van Zuijlen G., Visser D., van Dam J.C., Frank J., Teixeira de Mattos M.J., Heijnen J.J., 2001, 'Improved Rapid Sampling for *in vivo* kinetics of Intracellular Metabolites in *Saccharomyces cerevisiae*', Biotech. Bioeng. 75, 406-415.

Lawson, J.W.R., Veech R.L., 1979, 'Effects of pH and Free Mg^{2+} on the K_{eq} of the Creatine Kinase Reaction and Other Phosphate Hydrolyses and Phosphate Transfer Reactions', The Journal of Biological Chemistry 254 (14), 6528-6537.

Luo B., Groenke K., Takors R., Wandrey C., Oldiges M., 2007, 'Simultaneous determination of multiple intracellular metabolites in glycolysis, pentose phosphate pathway and tricarboxylic acid cycle by liquid chromatography–mass spectrometry', Journal of Chromatography A 1147, 153-164.

Maharjan, R.P., Ferenci T., 2003, 'Global metabolite analysis: the influence of extraction methodology on metabolome profiles of *Escherichia coli*', Analytical Biochemistry 313, 145-154.

Mashego, M.R., Rumbold K.,de Mey M., Vandamme E., Soetaert W.,Heijnen J.J., 2007, 'Microbial metabolomics: past, present and future methodologies', Biotechnol Lett 29, 1-16.

Mashego, M.R., Wu L., van Dam J.C., Ras C., Vinke J.L., van Winden W.A., van Gulik W.M., Heijnen J.J., 2004, 'MIRACLE: Mass Isotopomer Ratio Analysis of U-13C-Labeled Extracts. A New Method for Accurate Quantification of Changes in Concentrations of Intracellular Metabolites', Biotech. Bioeng. 85, 620-628.

Mashego, M.R., van Gulik W.M., Vinke J.L., Heijnen J.J., 2003, 'Critical Evaluation of Sampling Techniques for Residual Glucose Determination in Carbon-Limited Chemostat Culture of *Saccharomyces cerevisiae*', Biotech. Bioeng. 83, 395-399.

Nanchen, A., Schicker A., Sauer U., 2006, 'Nonlinear Dependency of Intracellular Fluxes on Growth Rate in Miniaturized Continuous Cultures of *Escherichia coli*', Applied and Environmental Microbiology 72 (2), 1164–1172.

Pramanik, J., Keasling J.D., 1997, 'Stoichiometric Model of *Escherichia coli* Metabolism: Incorporation of Growth-Rate Dependent Biomass Composition and Mechanistic Energy Requirements',Biotechnology and Bioengineering 56(4), 398-421.

Reed J.L., Vo T.D., Schilling C.H., Palsson B.O., 2003, 'An expanded genome-scale model of *Escherichia coli* K-12 (*i*JR904GSM/GPR)', Genome Biology 4, R54.

Schaefer, U., Boos W., Takors R., Weuster-Botz D., 1999, 'Automated Sampling Device for Monitoring Intracellular Metabolite Dynamics' , Analytical Biochemistry 270, 88-96.

Schaub, J., Schiesling C., Reuss M., Dauner M., 2006, 'Integrated Sampling Procedure for Metabolome Analysis', Biotechnol. Prog. 22, 1434-1442.

Seeholzer, S.H., 1993, 'Phosphoglucose isomerase: A ketol isomerase with aldol C2-epimerase activity', Proc. Nati. Acad. Sci. USA 90, 1237-1241.

Theobald, U., Mailinger W., Baltes M., Rizzi M., Reuss M., 1997, '*In Vivo* Analysis of Metabolic Dynamics in *Saccharomyces cerevisiae*: I. Experimental Observations ', Biotechnol Bioeng 55, 305-316.

Theobald, U., Mailinger W., Reuss M., Rizzi M., 1993, '*In vivo* analysis of glucose-induced fast changes in yeast adenine nucleotide pool applying a rapid sampling technique', Analytical Biochemistry 214 (1), 31-37.

van Dam, J.C., Eman M.R., Frank J., Lange H.C., van Dedem G.W.K., Heijnen J.J., 2002, 'Analysis of glycolytic intermediates in *Saccharomyces cerevisiae* using anion exchange chromatography and electrospray ionization with tandem mass spectrometric detection', Analytica Chimica Acta 460, 209-218.

Verduyn C., Postma E., Scheffers W.A., Vandijken J.P., 1992, 'Effect of benzoic acid on metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration and alcoholic fermentation', Yeast 8(7):501–517.

Villas-Boas, S.G., Bruheim P., 2007, 'Cold glycerol–saline: The promising quenching solution for accurate intracellular metabolite analysis of microbial cells', Analytical Biochemistry 370, 87-97.

Villas-Boas S.G., Hojer-Pedersen J., Akesson M., Smedsgaard J. , Nielsen J., 2005, 'Global metabolite analysis of yeast: evaluation of sample preparation methods', Yeast 22, 1155-1169.

Visser D., van Zuylen G.A., van Dam J.C., Oudshoorn A., Eman M.R., Ras C., van Gulik W.M., Frank J., van Dedem G.W., Heijnen J.J., 2002, 'Rapid Sampling for Analysis of *in vivo* kinetics using the BioScope: A system for continuous-pulse experiments', Biotechnol Bioeng. 79 (6), 674-681.

Wilcock, A.R., Goldberg D.M., 1972, 'Kinetic Determination of Malate Dehydrogenase Activity Eliminating Problems Due to Spontaneous Conversion of Oxaloacetate to Pyruvate', Biochemical Medicine 6, 116-126

Wittmann, C., Kromer J.O., Kiefer P., Binz T., Heinzle E., 2004, 'Impact of the cold shock phenomenon on quantification of intracellular metabolites in bacteria', Analytical Biochemistry 327, 135-139.

Wold, F., Ballou C.E., 1957, 'Studies on the enzyme enolase. I. Equilibrium studies', J Biol Chem. 227(1), 301-312

Wu L., van Dam J., Schipper D., Kresnowati M.T., Proell A.M., Ras C., van Winden W.A., van Gulik W.M., Heijnen J.J., 2006, Short-term metabolome dynamics and carbon, electron, and ATP balances in chemostat-grown *Saccharomyces cerevisiae* CEN.PK 113-7D following a glucose pulse. Appl Environ Microbiol. 72(5):3566-3577.

Wu L., Mashego M. R., van Dam J. C., Proell A. M., Vinke J.L., Ras C., van Winden W. A., van Gulik W.M., Heijnen J. J., 2005, 'Quantitative analysis of the microbial metabolomics by isotope dilution mass spectrometry using uniformly ^{13}C-labeled cell extracts as internal standards', Anal. Biochem. 336: 164-171.

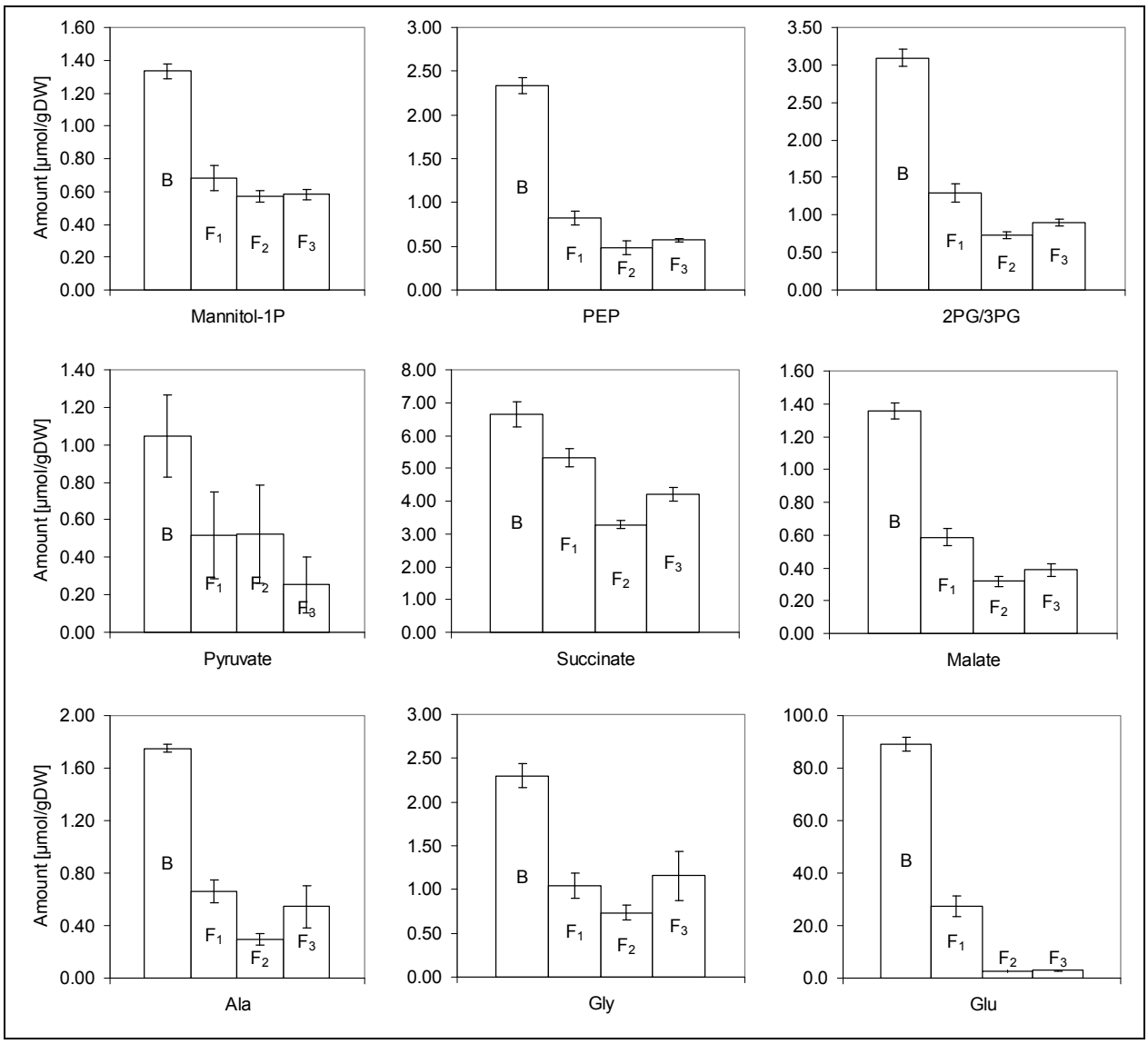

Figure 1: Some examples of measured steady state amounts of metabolites and amino acids in the broth (B) and in the culture filtrates obtained with three different methods: (F_1) quenching of the broth sample with cold stainless steel beads and subsequent filtration, (F_2) direct filtration of the broth sample without cooling and (F_3) direct centrifugation of the broth. Bars represent the averages with their standard errors of four replicate samples taken from two independent chemostat experiments, analyzed in duplicate.

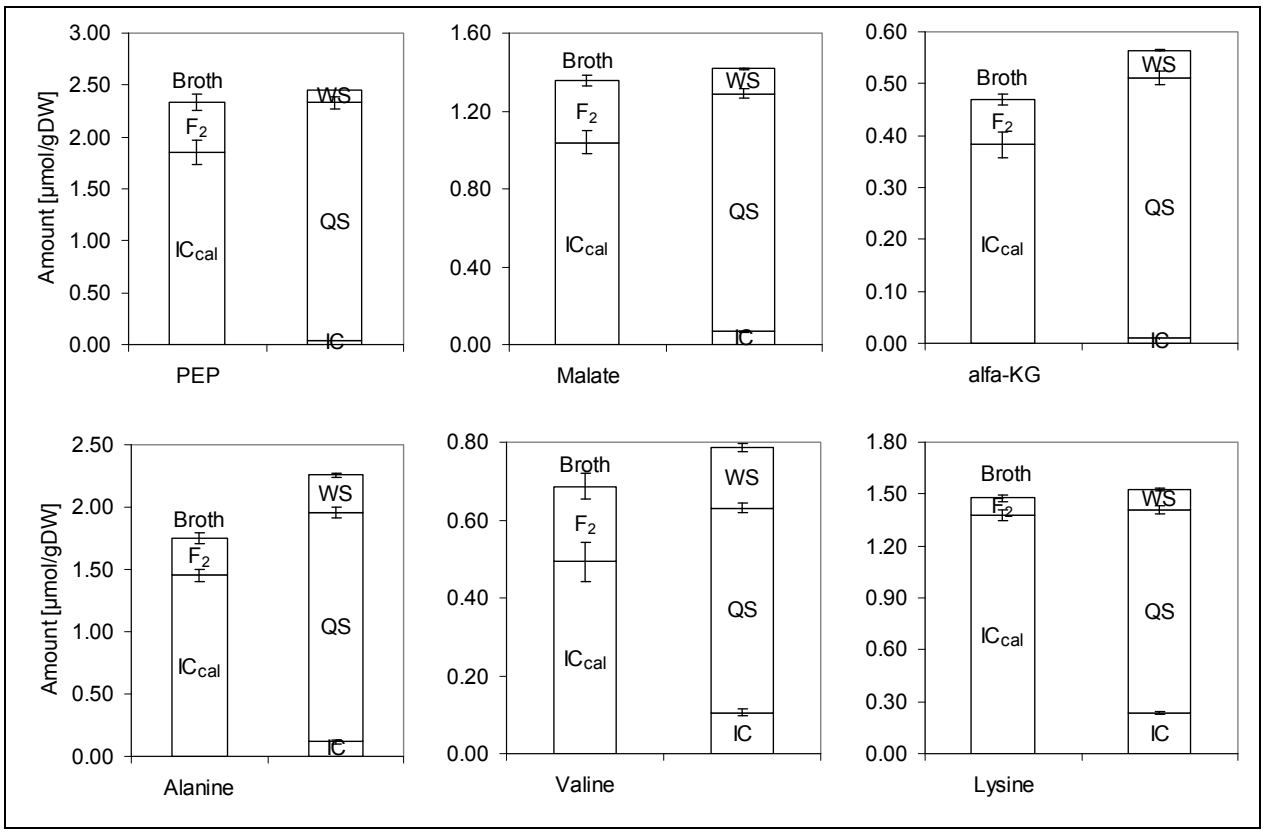

Figure 2: Examples of results of the balancing approach for quantification of metabolite leakage during the cold methanol quenching procedure: (F_2) amount measured in the filtrate, (IC_{cal}=B-F_2) calculated amount in the cell pellet, (WS) measured amount in the washing solution, (QS) measured amount in the quenching solution, (IC) measured amount in the biomass pellet. Bars represent the averages, with their standard errors, of four replicate samples taken from two independent chemostat experiments, analyzed in duplicate.

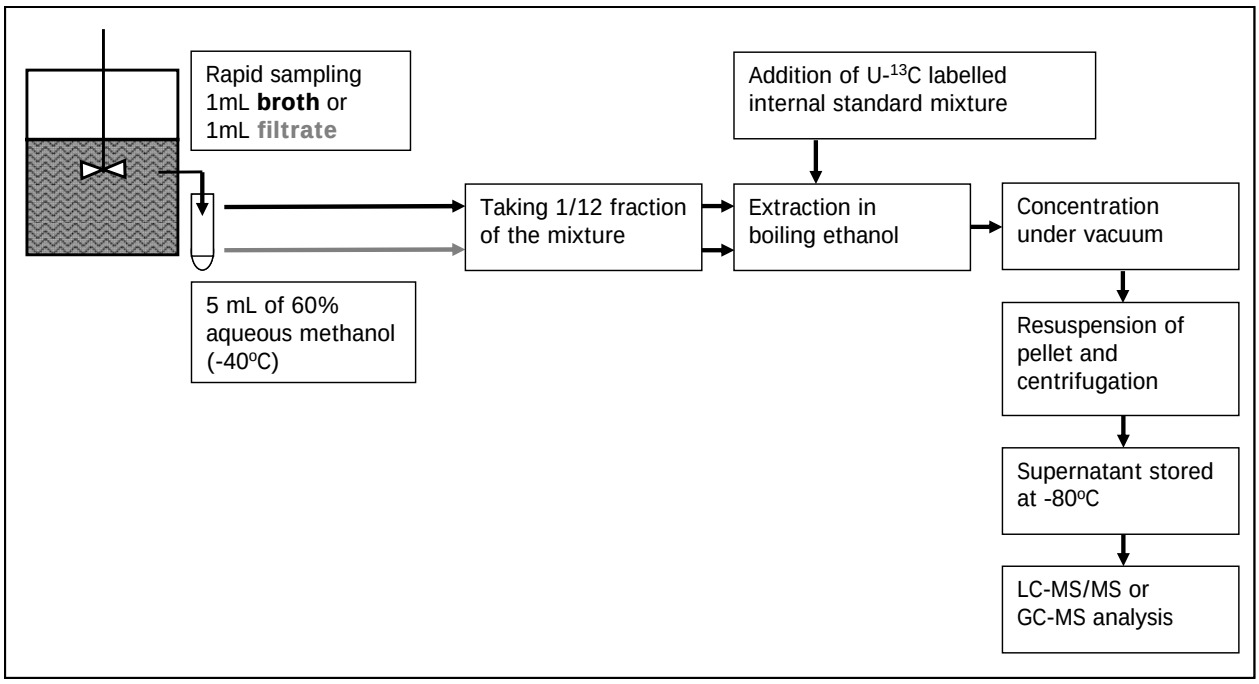

Figure 3: The applied differential sampling and analysis procedure for accurate metabolome analysis in *E. coli*

740 **Table 1:** The methods used for determination of intracellular metabolites in aerobic glucose-limited chemostat grown *Escherichia coli* K12 cells

	Schaefer *et al*., 1999	**Buchholz *et al*., 2001**	**Buchholz *et al*., 2002**	**Chassagnole *et al*., 2002**	**Schaub *et al*., 2006**	**Hiller *et al*., 2007b**
***E. coli* Strain**	K12 DSM 498	K 12	K 12	K12 W3110	K12 W3110	K12
Dilution rate [h^{-1}] (Reactor volume, T)	0.125 (7 L, 37°C)	0.125 (0.4 L, ?)	0.125 (7 L, 37°C)	0.1 (1.5 L, 35°C)	0.1 (1.5 L)	0.125 (3 L, ?)
Biomass conc [gDW/L]	10.7	10.0	10.0	8.7	2.3	10.0 ?
Sampling	Rapid sampling device 5 mL broth	Pre-cooled syringes 5 mL broth	Schaefer *et al*., 1999 5 mL broth	Buziol *et al*., 2002	Heat exchanger	- 6 g broth
Quenching	60%(v/v) methanol (-50°C) (15 mL)	60%(v/v) methanol + 70 mM HEPES (-50°C) (15 mL)	60%(v/v) methanol + 70 mM HEPES (-50°C) (15 mL)	Liquid nitrogen or Perchloric acid (-25 °C, %35w/w)	Heat exchanger	60%(v/v) methanol + 30 mM TEA (-40°C) (25 g)
Separation	Centrifugation (-20°C)	Centrifugation (-20°C)	Centrifugation (-20°C)	No	No	Centrifugation (-19°C)
Washing	No	No	No	No	No	No
Internal standard	No	0.5 mM cGMP	0.5 mM cGMP	No	No	Std mixture
Extraction	Perchloric acid (2 mL) (-18 °C, %35w/v)	Perchloric acid (2 mL) (%35w/v)	Perchloric acid (%35v/v)	Tris-H_2SO_4/EDTA (0.5 sv) KOH (0.3N, -25 °C) Perchloric acid (-25 °C, %35w/w)	Heat exchanger	water + 30 mM TEA (95°C) (4 mL)
Concentration	No	No	No	No	No	Lyophilization
Analysis Method	Enzymatic/ HPLC	ESI LC-MS	Enzymatic/HPLC/ LC-MS	Enzymatic/ HPLC	IC-MS	Enzymatic/LC-MS

741

742

743 **Table 2:** Average net conversion rates and carbon and redox recoveries of the steady state aerobic glucose-limited chemostat cultivations of *E. coli*,
744 carried out at D=0.1 h^{-1} in two different chemostat systems

Chemostat Volume	**μ^* [mmol/Cmol.h]**	**$-q_S$ [mmol/Cmol.h]**	**$-q_{O2}$ [mmol/Cmol.h]**	**q_{CO2} [mmol/Cmol.h]**	**q_{bp} [mmol/Cmol.h]**	**Carbon Balance**	**Redox Balance**
4 L	123.75 ± 4.93	38.29 ± 2.90	93.87 ± 3.44	97.14 ± 7.85	20.55 ± 1.71	96.1 %	97.4 %
0.5 L	121.91 ± 4.12	38.98 ± 2.22	96.61 ± 3.28	101.45 ± 6.00	19.31 ± 2.54	95.5 %	96.0 %

745 $^*\mu=q_X=D+q_{bp}$

Table 3: The steady-state concentrations and amounts of some metabolites, amino acids and adenine nucleotides in the filtrate (F_2), in the total broth (B), the percentage of metabolites in the filtrate, and intracellular amounts in aerobic glucose limited chemosat cultures grown at D= 0.1 h^{-1}. The indicated errors represent the standard errors of the mean.

	F_2 [µM]	F_2 [µmol/gDW]	B [µmol/gDW]	F_2/B*100 [%]	IC_{cal} [µmol/gDW]
Central Metabolites					
G6P	1.33	0.13 ± 0.02	1.54 ± 0.05	8.1	1.42 ± 0.06
F6P	1.12	0.11 ± 0.04	0.48 ± 0.01	21.9	0.38 ± 0.04
T6P	0.59	0.06 ± 0.004	0.19 ± 0.01	29.2	0.13 ± 0.01
M6P	0.12	0.01 ± 0.003	0.49 ± 0.01	2.2	0.48 ± 0.01
6PG	4.19	0.39 ± 0.05	0.49 ± 0.01	80.4	0.10 ± 0.05
Mannitol-1P	8.51	0.80 ± 0.08	1.79 ± 0.03	44.8	0.99 ± 0.09
G3P	1.58	0.15 ± 0.02	0.32 ± 0.02	46.7	0.17 ± 0.02
FBP*			0.82 ± 0.04		
F2,6bP	0.68	0.06 ± 0.01	0.41 ± 0.01	15.5	0.35 ± 0.01
2PG+3PG	6.06	0.57 ± 0.04	2.22 ± 0.03	25.7	1.65 ± 0.05
PEP	5.44	0.51 ± 0.04	2.12 ± 0.03	24.1	1.61 ± 0.05
Pyruvate	5.18	0.49 ± 0.06	1.24 ± 0.07	39.3	0.75 ± 0.10
α-ketoglutarate	0.68	0.06 ± 0.005	0.38 ± 0.01	17.0	0.31 ± 0.01
Succinate	20.71	1.95 ± 0.28	4.60 ± 0.14	42.4	2.65 ± 0.31
Fumarate	0.20	0.02 ± 0.002	0.24 ± 0.01	7.9	0.22 ± 0.01
Malate	3.98	0.38 ± 0.02	1.32 ± 0.02	28.5	0.94 ± 0.03
Amino Acids					
Alanine	2.33	0.22 ± 0.01	1.55 ± 0.03	14.1	1.34 ± 0.03
Asparagine	1.04	0.10 ± 0.01	0.68 ± 0.01	14.4	0.58 ± 0.01
Aspartate	2.77	0.26 ± 0.01	2.83 ± 0.03	9.2	2.57 ± 0.03
Glutamate	16.53	1.56 ± 0.09	76.25 ± 0.70	2.0	74.69 ± 0.71
Glutamine	2.30	0.22 ± 0.02	6.36 ± 0.06	3.4	6.14 ± 0.06
Glycine	5.57	0.52 ± 0.03	2.04 ± 0.05	25.7	1.51 ± 0.06
Histidine	1.21	0.11 ± 0.005	0.26 ± 0.01	44.0	0.15 ± 0.01
Isoleucine	0.55	0.05 ± 0.004	0.17 ± 0.01	31.6	0.11 ± 0.01
Leucine	0.72	0.07 ± 0.01	0.43 ± 0.02	15.9	0.36 ± 0.02
Lysine	0.84	0.08 ± 0.01	1.29 ± 0.02	6.1	1.21 ± 0.02
Methionine	0.19	0.02 ± 0.002	0.07 ± 0.002	25.6	0.05 ± 0.003
Phenylalenine	1.91	0.18 ± 0.02	0.31 ± 0.02	58.9	0.13 ± 0.03
Proline	0.90	0.08 ± 0.01	0.74 ± 0.01	11.4	0.66 ± 0.01
Serine	5.54	0.52 ± 0.06	1.05 ± 0.06	49.5	0.53 ± 0.08
Threonine	1.97	0.19 ± 0.01	0.65 ± 0.01	28.4	0.47 ± 0.02
Tryptophan	0.21	0.02 ± 0.002	0.04 ± 0.001	47.8	0.02 ± 0.003
Tyrosine	0.90	0.08 ± 0.004	0.27 ± 0.01	31.4	0.18 ± 0.01
Valine	1.30	0.12 ± 0.01	0.63 ± 0.04	19.5	0.51 ± 0.04
Ornithine	1.84	0.17 ± 0.01	0.66 ± 0.02	26.2	0.49 ± 0.03
Adenine nucleotides					
ATP	≤0.01	≤9.43E-4	5.95 ± 0.06	0	5.95 ± 0.06
ADP	≤2.50E-4	≤2.35E-5	2.31 ± 0.01	0	2.31 ± 0.01

AMP	≤0.01	≤9.43E-4	0.91 ± 0.02	0	0.91 ± 0.02

[*]FBP was quantified in the broth only.

Table 4: Turnover times (τ) of some metabolites and amino acids, calculated from the measured pool sizes (see Table 3) and the results of the metabolic flux balancing procedure.

	τ [s]
Central Metabolites	
G6P	3.6
F6P	1.2
6PG	1.1
G3P	13.1
2PG+3PG	1.9
PEP	2.7
Pyruvate	1.5
α-ketoglutarate	0.6
Succinate	8.9
Fumarate	0.7
Malate	2.8
Amino Acids	
Alanine	76.7
Asparagine	81.7
Aspartate	35.0
Glutamate	229.0
Glutamine	80.0
Glycine	31.0
Histidine	53.8
Isoleucine	12.9
Leucine	27.1
Lysine	119.7
Methionine	10.5
Phenylalenine	23.8
Proline	101.4
Serine	8.0
Threonine	29.3
Tryptophan	11.9
Tyrosine	44.3
Valine	40.9
Ornithine	49.1

Table 5: Mass action ratios (MAR) calculated from the measured metabolite levels and published equilibrium constants for some relevant equilibrium reactions.

Enzyme	Reaction	MAR	K_{eq}	Reference
PGI	G6P ⇌ F6P	0.27 ± 0.03	0.33	Seeholzer 1993
PMI	F6P ⇌ M6P	1.27 ± 0.14	1.10	Seeholzer 1993
PGM+ENO*	2+3PG ⇌ PEP	0.98 ± 0.04	K_{PGM}=0.19	Grisolia and Carreras,1975
			K_{ENO}=5.00	Wold and Ballou, 1957
AK	2ADP ⇌ AMP +ATP	1.01 ± 0.03	0.57-1.06	Lawson and Veech, 1979
FUM	Malate ⇌ Fumarate + H_2O	0.23 ± 0.01	0.23	Keruchenko *et al*., 1992

$$^{*}\frac{PEP}{2PG+3PG}=\left(\frac{1}{K_{ENO}}+\frac{1}{K_{ENO}K_{PGM}}\right)^{-1}=0.80$$