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ORIGINAL ARTICLE

Optimization of cold methanol quenching for quantitative metabolomics of *Penicillium chrysogenum*

Lodewijk P. de Jonge · Rutger D. Douma · Joseph J. Heijnen · Walter M. van Gulik

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Abstract A sampling procedure for quantitative metabolomics in *Penicillium chrysogenum* based on cold aqueous methanol quenching was re-evaluated and optimized to reduce metabolite leakage during sample treatment. The optimization study included amino acids and intermediates of the glycolysis and the TCA-cycle. Metabolite leakage was found to be minimal for a methanol content of the quenching solution (QS) of 40% (v/v) while keeping the temperature of the quenched sample near −20°C. The average metabolite recovery under these conditions was 95.7% (±1.1%). Several observations support the hypothesis that metabolite leakage from quenched mycelia of *P. chrysogenum* occurs by diffusion over the cell membrane. First, a prolonged contact time between mycelia and the QS lead to a somewhat higher extent of leakage. Second, when suboptimal quenching liquids were used, increased metabolite leakage was found to be correlated with lower molecular weight and with lower absolute net charge. The finding that lowering the methanol content of the quenching liquid reduces metabolite leakage in *P. chrysogenum* contrasts with recently published quenching studies for two other eukaryotic micro-organisms. This demonstrates that it is necessary to validate and, if needed, optimize the quenching conditions for each particular micro-organism.

Keywords Quantitative metabolomics · Intracellular metabolites · Cold methanol quenching · Metabolite leakage · *Penicillium chrysogenum*

Abbreviations

2PG	2-Phosphoglycerate
3PG	3-Phosphoglycerate
6PG	6-Phosphogluconate
ATP	Adenosine triphosphate
E4P	Erythrose 4-phosphate
EDTA	Ethylenediaminetetraacetic acid
F6P	Fructose 6-phosphate
FBP	Fructose 1,6-bisphosphate
G1P	Glucose 1-phosphate
G3P	Glycerol 3-phosphate
G6P	Glucose 6-phosphate
g_{DW}	Gram of dry weight biomass
M1P	Mannose 1-phosphate
M6P	Mannose 6-phosphate
PAA	Phenylacetic acid
PEP	Phosphoenolpyruvate
R5P	Ribose 5-phosphate
S7P	Sedoheptulose 7-phosphate
T6P	Trehalose 6-phosphate
αKG	α-Ketoglutarate

1 Introduction

Quantitative metabolome analysis is an important tool in microbial systems biology and metabolic engineering, such as for the determination of in vivo kinetic parameters and in isotopic nonstationary ^{13}C flux analysis (Nöh et al. 2007; Schaub et al. 2008). Metabolomics of micro-organisms

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L. P. de Jonge · R. D. Douma · J. J. Heijnen · W. M. van Gulik (✉)
Department of Biotechnology, Kluyver Centre for Genomics of Industrial Fermentation, Delft University of Technology, Julianalaan 67, 2628 BC Delft, the Netherlands
e-mail: w.m.vangulik@tudelft.nl

involves a number of steps. These include at least sampling, quenching of metabolic activity, metabolite extraction from the cells and quantification, but may include additional sample processing steps, such as washing of cells to prevent interference of the exometabolome, and sample concentration (Mashego et al. 2007; Álvarez-Sánchez et al. 2010).

The turnover time of many intermediates of both primary and secondary metabolism of micro-organisms is in the range of sub seconds to several tens of seconds (Weibel et al. 1974; Taymaz-Nikerel et al. 2009; Douma et al. 2010). Sampling and quenching of metabolic activity should therefore be sufficiently rapid to prevent (inter)conversion. Several rapid sampling devices and protocols have been developed in the past decades to achieve sub second sampling times (Schädel and Franco-Lara 2009; van Gulik 2010). Fast quenching of metabolic activity in cold aqueous methanol (De Koning and Van Dam 1992) has become very popular, because it has the advantage that, provided that the cells remain intact, it allows washing of the cells to remove extracellular metabolites by centrifugation or filtration. This is required if the extracellular amounts of metabolites are significant compared to the corresponding intracellular amounts, to prevent overestimation of the intracellular amounts (Bolten et al. 2007; Taymaz-Nikerel et al. 2009; Douma et al. 2010).

Nevertheless, it has become apparent that contact of cells with cold aqueous methanol will not seldom lead to a loss of metabolites from the cells (Wittmann et al. 2004; Villas-Bôas et al. 2005; Bolten et al. 2007; Faijes et al. 2007; Canelas et al. 2008; Sellick et al. 2009; Carnicer et al. 2011). From the studies performed on this subject so far, it seems that the underlying mechanism is different for prokaryotes and eukaryotes. For the former, the sudden (<1 s) change in temperature alone appears to be enough to induce release of intracellular metabolites (Wellerdiek et al. 2009), which is therefore usually referred to as the cold shock phenomenon. Despite some conflicting reports (De Koning and Van Dam 1992; Gonzalez et al. 1997; Hajjaj et al. 1998), metabolite leakage during cold methanol quenching was also clearly demonstrated for the eukaryote *Saccharomyces cerevisiae* (Villas-Bôas et al. 2005; Canelas et al. 2008). The loss of metabolites from these eukaryotic cells seems to occur through a process of diffusion over the cell membrane, in which the time of exposure, quenching temperature, properties of the cold aqueous methanol solution (e.g. ionic strength) and physicochemical properties of the metabolites (e.g. size and polarity) are factors that determine the extent of leakage (Canelas et al. 2008).

Recently a protocol for quantitative metabolomics in *Penicillium chrysogenum* relying on cold aqueous methanol quenching was published (Nasution et al. 2006). Until now this protocol has not been evaluated for a large set of metabolites. The aim of our study was to critically evaluate the applicability of cold methanol quenching for quantitative metabolomics of *P. chrysogenum*. Hereby a mass balance based approach was used as proposed by Canelas et al. (2008). For this, the fate of a large set of metabolites with various physicochemical properties was followed during sample treatment by analyzing different samples and sample fractions, including quenched total broth (TB) samples, quenched and washed cell pellets and supernatants.

2 Materials and methods

2.1 Solvents and chemicals

HPLC-grade methanol and ethanol were obtained from Baker (The Netherlands). Analytical grade standards were obtained from Sigma-Aldrich.

2.2 Strain

A strain of *P. chrysogenum* (DS17690) with a high penicillin yield was kindly donated as spores from a culture grown on rice grains by DSM Anti-Infectives (Delft, The Netherlands). This strain has been well characterized in terms of its productivity and yields during chemostat cultivation (Nasution et al. 2006; van Gulik et al. 2000).

2.3 Media and chemostat cultivations

The batch medium contained per l of demineralized water: 15.0 g glucose, 5.0 g $(NH_4)_2SO_4$, 1.0 g KH_2PO_4, 0.5 g $MgSO_4$, 0.41 g PAA and 2 ml of trace element solution. The trace element solution contained per l 75.0 g $Na_2EDTA{\cdot}2H_2O$, 10.0 g $ZnSO_4{\cdot}7H_2O$, 10.0 g $MnSO_4{\cdot}H_2O$, 20.0 g $FeSO_4{\cdot}7H_2O$, 2.5 g $CaCl_2{\cdot}2H_2O$, 2.5 g $CuSO_4{\cdot}5H_2O$. The pH of the trace element solution was set to 6.0 with NaOH pellets. All batch medium components except the glucose were dissolved in 3.6 l demineralized water. The pH was set to 5.6 and the solution was sterilized for 40 min at 121°C. The glucose was dissolved separately and demineralized water was added to bring the weight of the solution to 300 g. This solution was sterilized for 40 min at 110°C. For inoculation, 10 g of rice grains were submerged in 100 ml demineralized water for one hour. The batch medium, glucose solution and inoculum were introduced aseptically into the reactor.

The composition of the chemostat medium was the same as that of the batch medium except that the concentration of PAA was 0.76 g/l PAA. The PAA concentration in the

batch and chemostat media were designed to achieve a (residual) concentration of approximately 3 mM, which is not limiting for penicillin production, nor inhibiting for cell growth (van Gulik et al. 2000). The required amount of PAA for 50 l of medium was dissolved in 4 l of demineralized water by continuous stirring while adding KOH pellets to set the pH to 5.6. This solution was sterilized in a 55 l vessel for 40 min at 121°C. All other medium components were dissolved in 46 l of demineralized water. After setting the pH to 5.6 with KOH pellets, this solution was added to the PAA solution by filter sterilization (Supor DCF 0.2 μm filters, Pall Gelman Sciences, East Hills, NY). This medium supported a steady state biomass concentration of about 6 g_{DW}/l.

Cultivations were carried out in a 7 l fermentor (Applikon, The Netherlands) with a working volume of 4 l under an aerobic glucose-limited regime at 25°C, a pH of 6.5 and a dilution rate of 0.05 h^{-1} as described by Nasution et al. (2006).

2.4 Sampling and sample treatment procedures

Samples for analysis of were taken essentially as described by Nasution et al. (2006). Samples of ±1 g of broth were quickly (±0.7 s) withdrawn from the reactor and sprayed into a tube containing a quenching liquid using a rapid sampling device (Lange et al. 2001). Three variations were made with respect to the quenching liquid. The sampling tubes were filled either with 5 ml −40°C 60% (v/v) aqueous methanol, 5 ml −40°C pure methanol or 10 ml −25°C 40% (v/v) aqueous methanol. After sampling the content of each tube was immediately (<1 s after sampling) mixed by vortexing (for 2–5 s, until a vortex was established) and directly placed back in the cryostat. The exact amounts of sample were determined by weighing. Subsequently, the tubes were centrifuged for 5 min at 4,800 *g* in a cooled centrifuge at −20°C using a swing-out rotor, precooled at −40°C. After decanting, the cell pellets were resuspended by vortexing in 5 ml of a washing liquid that had the same temperature and composition as the quenching liquid and the tubes were centrifuged again. The supernatants of the first [from now on called quenching solution (QS)] and second [washing solution (WS)] centrifugation step were collected and weighed. The cell pellets, quenching and washing solutions were placed in the cryostat until further treatment. 100 μl of a ^{13}C internal standard solution (0°C) was added to the washed cell pellets to compensate for losses and degradation of metabolites during further sample treatment and for accurate quantification purposes by IDMS (Wu et al. 2005). The ^{13}C internal standard solution contained all relevant metabolites as U-^{13}C-labeled isotopologues and was obtained from a *P. chrysogenum* fed-batch culture grown on >99% U-^{13}C-labeled glucose (Campro Scientific, Veenendaal, the Netherlands) and ^{13}C-labeled PAA with all carbon atoms of the aromatic ring labeled (Sigma-Aldrich). The washed cell pellets containing the ^{13}C internal standards were then extracted using boiling 75% (v/v) ethanol and further treated as described earlier (Nasution et al. 2006).

300–500 μl of the collected QS and WS were transferred to clean tubes. Subsequently 100 μl of the ^{13}C internal standard solution was added to each of them. The QS and WS samples were subjected to the boiling ethanol treatment to denature possible enzymes present in the supernatant, thereby preventing conversion of metabolites. These samples were further processed in the same way as the cell pellet samples.

Total broth (TB) samples were taken by sampling ±1 g of broth in 5 ml −40°C 60% (v/v) aqueous methanol. After weighing, 300 μl of the homogenized suspension was transferred to clean tubes and, after addition of 100 μl of the ^{13}C internal standard solution, the broth samples were subjected to the boiling ethanol treatment and were further processed in the same way as the cell pellets. Broth samples for quantification of extracellular metabolite levels (EX) were immediately cooled to 0°C and filtered to remove the cells, using the cold steel bead method (Mashego et al. 2004) with the difference that the filtrate was directly injected into a 5 ml solution of −40°C 60% (v/v) aqueous methanol and vortexed thoroughly. Subsequently 300 μl of this mixture was combined with 100 μl of the ^{13}C internal standard solution, subjected to ethanol boiling and further treated in the same way as the cell pellets. In the second experiment investigating the effect of quenching time (see Sect. 3.2), TB and EX samples were taken in 10 ml of −25°C 40% (v/v) aqueous methanol instead of in 5 ml of −40°C 60% (v/v) aqueous methanol.

2.5 Analysis

Three different analytical platforms were used. GC–MS was used to analyze the free amino acid pools of ornithine and the twenty proteinogenic amino acids except arginine, cysteine and valine, by using the EZ:Faast kit for free amino acid analysis from Phenomenex (Torrance, CA, USA). In one experiment, anion-exchange LC–ESI–MS/MS was used for the analysis of G6P, F6P, M6P, FBP, T6P, 6PG, PEP G3P, pyruvate, αKG, succinate, fumarate, malate, and the combined pools of citrate + isocitrate, 2PG + 3PG and G1P + M1P (van Dam et al. 2002). In a second experiment, another GC–MS method was used for the analysis of G6P, F6P, M6P, FBP, T6P, PEP, pyruvate, αKG, succinate, fumarate, malate, citrate, isocitrate, 2PG, 3PG, R5P, S7P and E4P (Cipollina et al. 2009). All analyses were performed at least in duplicate and quantification of the metabolites was based on the use of U-^{13}C-labeled

cell extract as internal standard (Mashego et al. 2004; Wu et al. 2005).

2.6 Mass balance calculations

Metabolite amounts were quantified in the above mentioned samples and sample fractions. Standard deviations were estimated from two (first experiment) or three (second experiment) replicate samples taken from the same chemostat culture. Because ^{13}C labeled internal standard mix was added to all sample fractions before the metabolite extraction procedure, possible partial degradation of metabolites was effectively corrected for. Therefore a mass balance can be established for every metabolite i and quenching protocol variation j which states that the metabolite amount measured in TB samples equals the sum of the amounts measured in cell pellets (IC), and quenching (QS) and washing supernatants (WS):

$$M_i(\mathrm{TB}) = M_{i,j}(\mathrm{IC}) + M_{i,j}(\mathrm{QS}) + M_{i,j}(\mathrm{WS}) \quad (1)$$

Furthermore, the amount which was released from cells into the quenching and washing solutions during sample treatment can be calculated from a second balance:

$$M_{i,j}(\mathrm{leakage}) = M_i(\mathrm{TB}) - M_i(\mathrm{EX}) - M_{i,j}(\mathrm{IC}) \quad (2)$$

with $M_{i,j}(\mathrm{leakage}) \geq 0$

If no metabolite leakage occurs, the metabolite amount in the cell pellet (IC) is equal to the difference between the amounts in TB samples and culture filtrate samples (EX). Leakage becomes evident when the amount in the cell pellet is smaller than the difference between TB and EX. Hence the inequality for the amount of leaking metabolites.

Since, except for the leakage term, all terms in both balances were measured, the data set contained redundant information, allowing statistical testing of the consistency of the measurements. For both experiments described in this study, this was done by calculating the χ^2 distributed consistency index h for each metabolite in a single protocol variation at a significance level of 0.05 (van der Heijden et al. 1994). Subsequently, the data that were not rejected by the test were reconciled. The reconciliation was achieved by least sqares minimization of the differences between measured and estimated metabolite amounts, weighed by their measurement errors and subjected to the constraints expressed by Eqs. 1 and 2 (Carnicer et al. 2011).

3 Results and discussion

3.1 Effect of methanol concentration

The first experiment aimed at evaluating the extent of metabolite leakage, when using the conventional QS (−40°C, 60% v/v aqueous methanol) as proposed in the protocol for quantitative metabolomics in *P. chrysogenum* of Nasution et al. (2006). In addition, two other quenching liquids were tested for comparison. Cold pure methanol (−40°C) was tested for the reason that it was reported to be the optimal quenching liquid for *S. cerevisiae* (Canelas et al. 2008), which is also a eukaryotic micro-organism. To further assess the effect of the methanol content, an aqueous solution with a lower methanol content of 40% (v/v) was tested too. Due to its higher freezing point, the 40% (v/v) aqueous methanol solution was precooled to −25°C instead of −40°C. To prevent that the temperature of the mixture of the broth sample and quenching liquid would rise above −20°C during sampling, a volume ratio of broth sample to quenching liquid of 1:10 (1 ml sample +10 ml quenching liquid) was used in this case. We considered −20°C as upper limit because Wellerdiek et al. (2009) showed that metabolic activity was absent at −20°C in the case of quenched *Corynebacterium glutamicum* cells.

The extent of leakage of metabolites from cells quenched in these three liquids was evaluated using a quantitative mass balance approach (see Sect. 2.6). As an example, the mass balances for the three applied quenching liquids and three selected metabolites with different physicochemical properties are shown in Fig. 1. Note that the metabolite amounts of all sample fractions are expressed in $\mu mol/g_{DW}$ to be able to compare them. In Fig. 1, the metabolite amounts measured in the quenched and washed cell pellet (IC, direct determination of the intracellular amount) should be compared to the amount calculated from the difference between the amounts in the TB and culture filtrate (TB − EX, indirect determination) which is considered to be the best estimate of the "true" intracellular amount. Obtaining the intracellular amount by this subtraction procedure is known in literature as the differential method (Bolten et al. 2007; Taymaz-Nikerel et al. 2009). From the results shown in Fig. 1 it can be seen that, when pure methanol was used as quenching liquid ("100%"), the amounts of fumarate and aspartate found in the cell pellet were considerably lower than the amounts obtained with the differential method (further on referred to as reference amount). Furthermore, for all three metabolites in this condition, as well as for aspartate in the "60%" condition, the sum of the amounts measured in the quenching and washing solutions were notably higher than the amounts measured in the culture filtrate. These observations indicate that metabolite leakage into the cold quenching liquid occurred under these conditions. Finally, it can be seen from this figure that no or hardly any G6P and fumarate leaked into the quenching liquids with 40 and 60% methanol. Note that the standard errors of the TB samples in Fig. 1 are large relative to those of the other sample fractions. This may be due to the fact that only a part of the

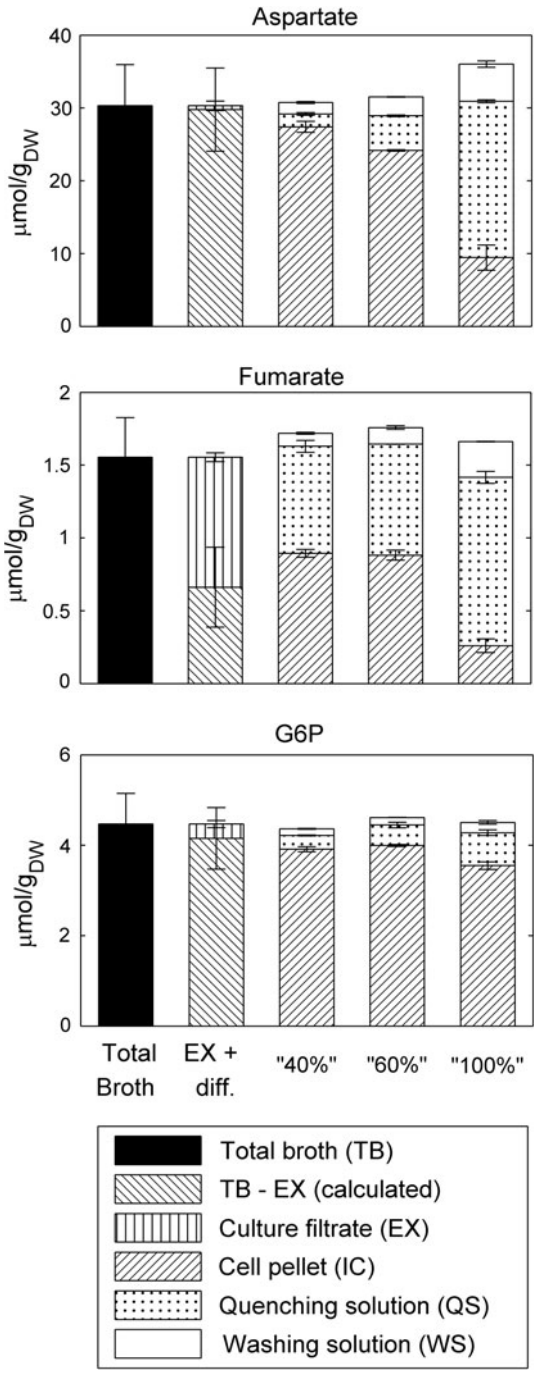

Fig. 1 Mass balances of G6P, aspartate and fumarate for the three protocol variations. "40%": quenching of 1 ml sample in 10 ml −25°C 40% (v/v) aqueous methanol; "60%": quenching of 1 ml sample in 5 ml −40°C 60% (v/v) aqueous methanol; "100%": quenching of 1 ml sample in 5 ml −40°C pure methanol. *Error bars* give the standard errors for duplicate samples, each analyzed in duplicate

quenched TB sample was used in the extraction step, which was done to limit the carry-over of sulfate and phosphate (originating from the medium), because too high concentrations of these salts interfere with the MS-based analysis (van Dam et al. 2002). An overview of the metabolite levels measured in the various sample fractions is given in the Supplementary Material (Table S1).

To verify the quality of the measurements, the consistency of the data was tested (see Sect. 2.6) to detect gross measurement errors. Only data that passed the test were included in the evaluation. For a better comparison of the results, those data were also reconciled under the constraints expressed in Eqs. 1 and 2.

To compare the performance of the three quenching liquids, the ratio of the metabolite amount in the cell pellet (IC) and the reference amount obtained by the differential method (TB − EX) was calculated for every metabolite for each of the three quenching liquids. These ratios can be considered as recoveries. For the complete set of evaluated metabolites, the average recoveries (±standard error) were 95.7% (±1.1%), 84.3% (±3.1%), and 49.8% (±6.6%) when quenching was performed using −25°C 40% (v/v) aqueous methanol, −40°C 60% (v/v) aqueous methanol and −40°C pure methanol, respectively. In Fig. 2a, the sets of metabolite-specific recoveries are represented by boxplots for all evaluated metabolites; on the left side, the results were calculated from the (consistent) raw data, and on the *right* side the recoveries were obtained from the reconciled data. Clearly, the best agreement between metabolite amounts found in the cell pellet and the reference amounts was obtained when −25°C 40% (v/v) aqueous methanol was used as the quenching liquid.

In Fig. 2b–d, the evaluated metabolites are separated into three classes, namely phosphorylated metabolites, organic acids and amino acids. In both the raw and the reconciled data it is observed that for all three compound classes the recoveries obtained by quenching in −25°C 40% (v/v) aqueous methanol were closest to 100%, while those obtained with −40°C pure methanol as quenching liquid were quite widely distributed. Especially the amounts of organic acids (Fig. 2c) and amino acids (Fig. 2d) recovered from the quenched and washed cell pellets were found to be reduced compared to the reference amounts when 60% (v/v) aqueous methanol or pure methanol was used for quenching. In the case of 40% (v/v) aqueous methanol, almost all metabolites had a recovery (calculated from reconciled data) higher than 90%, except for Gln (88.7%) and Gly (72.5%). All in all, it was concluded that −25°C 40% (v/v) aqueous methanol was the best performing of the three quenching liquids tested, although for some metabolites, especially amino acids, it did not completely prevent leakage.

The present finding that higher recoveries are obtained when the methanol fraction in the quenching liquid is reduced to 40%, contrasts with the findings of leakage studies performed on two other eukaryotic microorganisms (Canelas et al. 2008; Carnicer et al. 2011; Tredwell

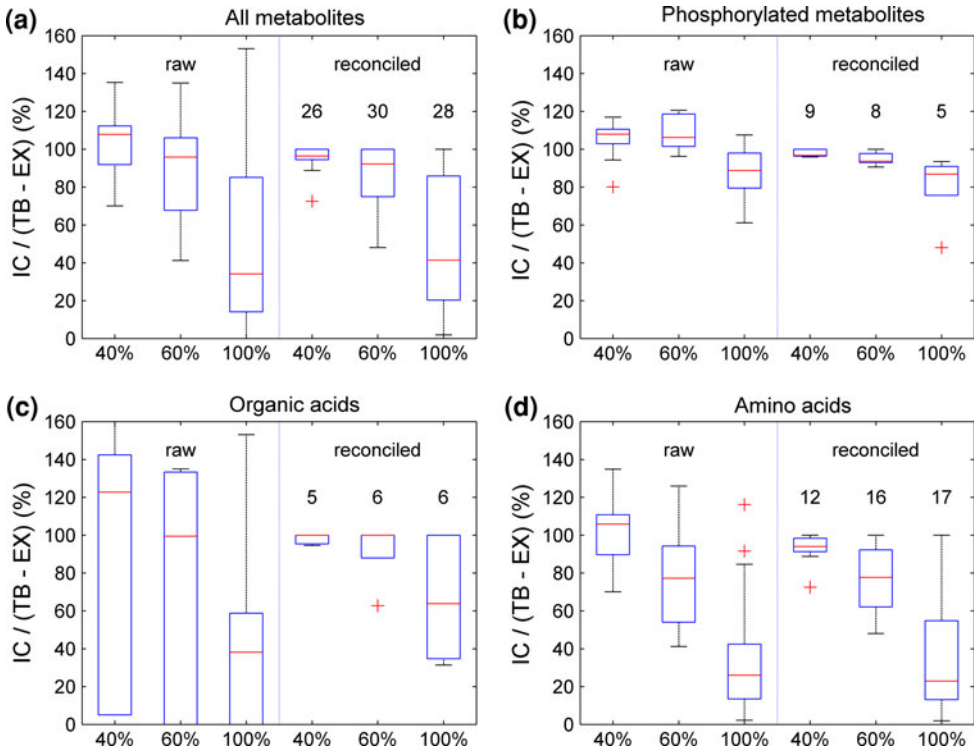

Fig. 2 Boxplots of the ratios between the intracellular amounts measured in quenched and washed cell pellets (IC) and the reference amounts calculated from the difference between the amounts measured in TB and culture filtrate (TB − EX) for the three protocol variations. "40%": quenching with 10 ml −25°C 40% (v/v) aqueous methanol; "60%": quenching with 5 ml −40°C 60% (v/v) aqueous methanol; "100%": quenching with 5 ml −40°C pure methanol. The boxplots are shown both for the consistent raw (*left*) and the reconciled (*right*) data and indicate the median, the first and third quartile. The whiskers indicate the most extreme point from the first and third quartile within a distance of 1.5 times the distance between the first and third quartile; points at a greater distance are indicated by crosses. The numbers above the boxplots on the *right* refer to the number of metabolites evaluated in that condition and apply also for the consistent raw data on the *left*. The four panels show results of **a** all evaluated metabolites, **b** only phosphorylated metabolites, **c** only organic acids and **d** only free amino acids

et al. 2011). For *S. cerevisiae* it was found that a higher methanol content and a lower temperature lead to a lower extent of leakage, with −80°C pure methanol being the optimal quenching liquid (Canelas et al. 2008). For cultures of *Pichia pastoris* it was found that the methanol content of the quenching liquid did not significantly affect the recovery of metabolites from cell pellets (Carnicer et al. 2011; Tredwell et al. 2011). These examples demonstrate that a dedicated validation of quenching liquids is required for every micro-organism before it is applied in a quantitative metabolomics study.

3.2 Prolonged exposure to quenching liquid

Results of a study with *S. cerevisiae* have suggested that leakage can occur by diffusion of metabolites over the cell membrane (Canelas et al. 2008). In that case, the extent of leakage would increase if quenched cells are not processed immediately, but are kept in the QS for a longer period of time. Therefore, in a second experiment, the degree of metabolite loss was evaluated for the situation that cells are exposed to the QS for a prolonged period of time. To study this, samples were taken from another chemostat culture operated under the same conditions. Using 10 ml −25°C 40% (v/v) aqueous methanol as the quenching liquid, one set of triplicate samples was processed immediately and another set was left in the cryostat at −25°C for 30 min before proceeding to the centrifugation and washing steps.

The results are summarized in Fig. 3. The average recoveries (±standard deviation) of the samples that were processed immediately ("0 min") and with a 30 min delay ("30 min") were 92.8% (±1.1%) and 88.0% (±1.3%), respectively (calculated from the reconciled data). The small difference between these averages and the distribution of the recoveries show that the extent of metabolite leakage increased due to the prolonged contact time with the quenching liquid. This finding supports the hypothesis that metabolite leakage can occur by diffusion over the cell membrane as suggested by Canelas et al. (2008). It also means that sample treatment should proceed as quickly as possible to the boiling ethanol step for extraction and enzyme inactivation.

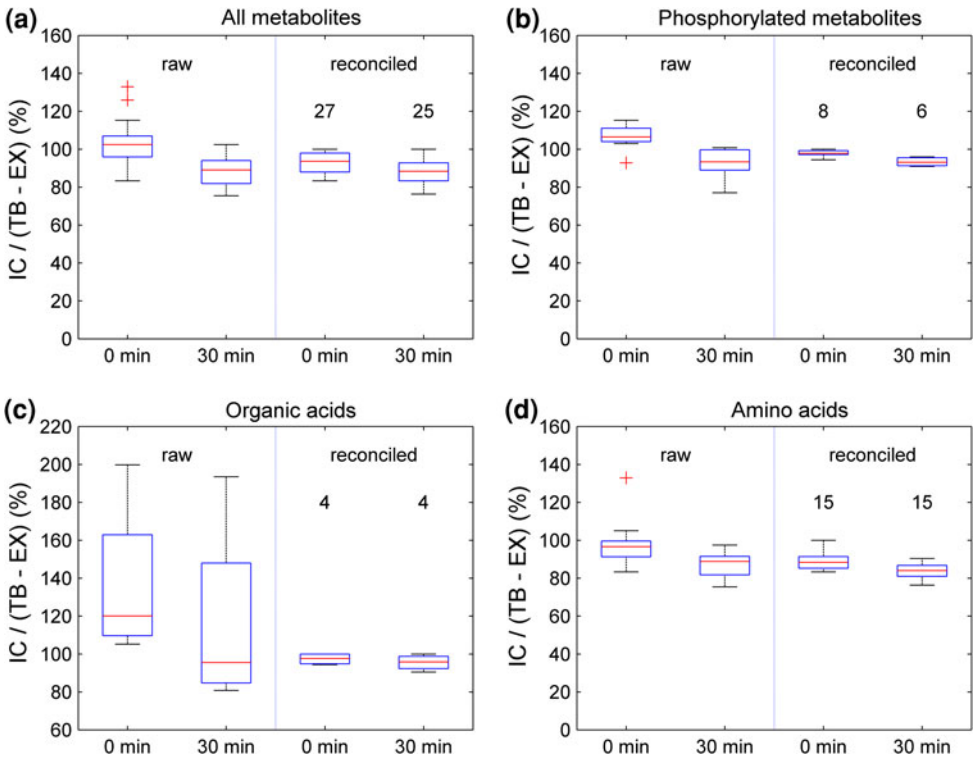

Fig. 3 Influence of the contact time of cells quenched with −25°C 40% (v/v) aqueous methanol on the extent of leakage. Samples were either processed immediately (0 min) or were left for 30 min in the QS at −25°C before being processed (30 min). The boxplots are plotted as in Fig. 2. The four panels show results of **a** all evaluated metabolites, **b** only phosphorylated metabolites, **c** only organic acids and **d** only free amino acids. Note that the range of the vertical axis in panel **c** was adjusted

3.3 Factors influencing the extent of metabolite leakage

Canelas et al. (2008) based their hypothesis that metabolite leakage in *S. cerevisiae* is driven by diffusion not only on the observation that the extent of leakage increased with quenching time, but also on the finding that smaller metabolites leaked more than larger ones, which corresponds with the fact that the diffusivity of smaller molecules is higher. We plotted the extent of leakage versus molecular weight for our data in Fig. 4a. Molecular weight was used as proxy for molecular size. The extent of leakage was calculated from the reconciled data as the percentage difference between the reference amount (TB − EX) and the amount in the cell pellets treated with 60% (v/v) aqueous methanol and pure methanol. Figure 4a shows the trend that smaller molecules leak more, which further supports the hypothesis that also in the case of *P. chrysogenum* leakage of metabolites from quenched biomass is driven by diffusion.

If metabolites leak from cells by diffusion, they have to pass the hydrophobic cell membrane, which is expected to be less likely to happen when they are electrically charged. Indeed a trend of a lower extent of leakage with higher absolute net charge can be observed in Fig. 4b. Here, the absolute net charge was calculated from the pKa values and an assumed intracellular pH of 7 (theoretical pKa values obtained from the software MarvinSketch 5.5.1.0 were used in case experimentally determined pKa values were not available, for example in the case of 2PG). The metabolites plotted as having an absolute net charge of close to 0 were zwitterionic free amino acids without a charged side-group but with a positively charged amino group and a negatively charged carboxyl group. The class of amino acids was found to show the largest extent of leakage in this study (see also Figs. 2d, 3d).

Canelas et al. (2008) observed that the extent of leakage from *S. cerevisiae* cells decreased when the methanol content of the quenching liquid was increased. Methanol is not as good a solvent as water for most metabolites, because they are mostly charged and polar. In combination with observations on the effects of the temperature and the ionic strength of the quenching liquid on metabolite leakage, they suggested that reduction of the solubility of the metabolites in the quenching liquid would decrease the extent of leakage. However, Carnicer et al. (2011) did not observe a significant difference in leakage for different methanol content of the cold aqueous methanol QS in case of *P. pastoris*, while we find that the extent of leakage from *P. chrysogenum* cells increases with an increase in the methanol content, which is the exact opposite of the finding of Canelas et al. (2008). These results make it doubtful whether solubility must be considered as a factor of importance in metabolite leakage.

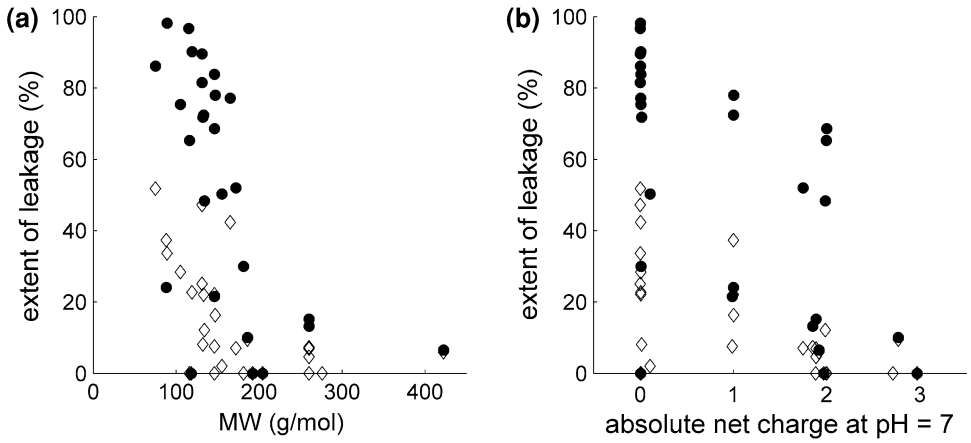

Fig. 4 Extent of leakage versus molecular weight (**a**) and versus absolute net charge (**b**). The extent of leakage was calculated from the reconciled data as the percentage difference between metabolite levels in the cell pellets and the reference amount (TB − EX). Each symbol represents the extent of leakage for one metabolite under the condition of using 60% (v/v) aqueous methanol (*open diamonds*) or pure methanol (*solid circles*) for quenching

3.4 The advantage of using a quantitative mass balance approach

In this work the extent of metabolite leakage during cold aqueous methanol quenching was evaluated using a quantitative mass balance approach. With this approach the amounts of metabolites are quantified in different sample fractions such that losses of metabolites from cells during methanol quenching can be detected and quantified. Several other studies aiming at minimization of leakage reported in literature only compared the amounts recovered from the cell pellet when using different quenching liquids (Villas-Bôas and Bruheim 2007; Spura et al. 2009). With such an approach it is possible to find the quenching procedure which results in the highest metabolite levels. However, the advantages of the mass balance approach are that the extent of leakage is also estimated and that it allows to check the consistency of the data. Furthermore, the use of ^{13}C labeled cell extract as internal standard in all samples and standards is recommended, because it corrects for partial degradation of metabolites during sample processing and storage and improves the precision of the mass spectrometric based quantification, as was also concluded by others (Büscher et al. 2009; Wellerdiek et al. 2009; Zamboni and Sauer 2009).

4 Concluding remarks and recommendations

For quantitative metabolomics studies in *P. chrysogenum*, −25°C 40% (v/v) aqueous methanol was found to be the optimal quenching and washing liquid of the three liquids compared in this work, because its use resulted in the highest recovery of metabolites from quenched and washed cell pellets. The volume ratio of sample to this quenching liquid should preferably be 1:10 to avoid that the temperature of the mixture rises above −20°C. Sample treatment should proceed as quickly as possible to metabolite extraction and definitive enzyme inactivation, because prolonged contact time between quenched cells and the quenching liquid can lead to increased metabolite losses.

Besides the contact time with the quenching liquid, the extent of leakage was found to depend on factors affecting the diffusivity, namely molecular weight and net charge, suggesting that metabolite leakage from quenched mycelia of *P. chrysogenum* is driven by diffusion.

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