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

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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 \(-20^\circ\text{C}\). 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
- gDW: 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
- aKG: a-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 \(^{13}\text{C}\) flux analysis (Nöh et al. 2007; Schaub et al. 2008). Metabolomics of micro-organisms
achieve sub second sampling times (Schaédel and Franco-
protocols have been developed in the past decades to
should therefore be sufficiently rapid to prevent
2010). Sampling and quenching of metabolic activity
et al. 1974; Taymaz-Nikerel et al. 2009; Douma et al.
the range of sub seconds to several tens of seconds (Weibel
mary and secondary metabolism of micro-organisms is in
ple concentration (Mashego et al. 2007; Álvarez-Sánchez
loss of metabolites from the cells (Wittmann et al. 2004;
compared to the corresponding intracellular amounts, to
metabolites by centrifugation or filtration. This is required
if the extracellular amounts of metabolites are significant
cold aqueous methanol (De Koning and Van
Hajjaj et al. 1998), metabolite leakage during cold meth-
(De Koning and Van Dam 1992; Gonzalez et al. 1997;
properties of the cold
induce release of intracellular metabolites (Wellerdiek
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relying on cold aqueous methanol
involves a number of steps. These include at least sam-
pling, quenching of metabolic activity, metabolite extrac-
tion from the cells and quantification, but may include
additional sample processing steps, such as washing of cells
to prevent interference of the exometabolome, and sam-
ples concentration (Mashego et al. 2007; Álvarez-Sánchez
et al. 2010).
The turnover time of many intermediates of both pri-
mary 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
Dame 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
(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 quantita-
tive 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 peni-
cillin 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 cul-
tivation (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 (NH4)2SO4, 1.0 g KH2PO4, 0.5 g
MgSO4, 0.41 g PAA and 2 ml of trace element solution.
The trace element solution contained per l 75.0 g Na2
EDTA-2H2O, 10.0 g ZnSO4-7H2O, 10.0 g MnSO4-H2O,
20.0 g FeSO4-7H2O, 2.5 g CaCl2-2H2O, 2.5 g CuSO4
5H2O. 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 13C 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 13C internal standard solution contained all relevant metabolites as U-13C-labeled isotopologues and was obtained from a P. chrysogenum fed-batch culture grown on >99% U-13C-labeled glucose (Campro Scientific, Veenendaal, the Netherlands) and 13C-labeled PAA with all carbon atoms of the aromatic ring labeled (Sigma-Aldrich). The washed cell pellets containing the 13C 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 13C 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 13C internal standard solution, was subjected to ethanol boiling and further treated 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 13C 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, zKG, 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, zKG, 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-13C-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):

$$M_i(TB) = M_{i,j}(IC) + M_{i,j}(QS) + M_{i,j}(WS)$$ (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:

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

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

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 $\chi^2$ 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 squares 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 ($-40^\circ\text{C}$, $60\%$ $v/v$ 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 ($-40^\circ\text{C}$) 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 $-25^\circ\text{C}$ instead of $-40^\circ\text{C}$. To prevent that the temperature of the mixture of the broth sample and quenching liquid would rise above $-20^\circ\text{C}$ 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 $-20^\circ\text{C}$ as upper limit because Wellerdiek et al. (2009) showed that metabolic activity was absent at $-20^\circ\text{C}$ 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 physico-chemical properties are shown in Fig. 1. Note that the metabolite amounts of all sample fractions are expressed in $\mu$mol/$\mu$gDW 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
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 box-plots 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
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 \(-80°C\)/C176C 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 \(-25°C\)/C176C 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.
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.
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-Boas 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*, $-25^\circ$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 $-20^\circ$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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