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ORIGINAL ARTICLE

Fast sampling for quantitative microbial metabolomics: new aspects on cold methanol quenching: metabolite co-precipitation

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Abstract The intra- and extracellular concentrations of 16 metabolites were measured in chemostat ($D = 0.1\ h^{-1}$) anaerobic cultures of the yeast *Saccharomyces cerevisiae* CEN.PK-113-7D growing on minimal medium. Two independent sampling workflows were employed: (i) conventional cold methanol quenching and (ii) a differential approach. Metabolites were quantified in different sample fractions (total, extracellular, quenching supernatant, methanol/water extract and pellet) in order to derive their mass balance. The differential method in combination with absolute metabolite quantification by gas-chromatography with isotope dilution mass spectrometry (GC–IDMS) was used as a benchmark to assess quality of the cold methanol quenching procedure. Quantitative comparison of metabolite concentrations in all fractions collected by different quenching techniques indicates asystematic loss of the total mass of various metabolites in course of the cold methanol quenching. Pellet resulting from the cold methanol quenching besides biomass contains considerable amounts of precipitated inorganic salts from the fermentation media. Quantitative analysis has revealed significant co-precipitation of polar extracellular metabolites together with these salts. This phenomenon is especially significant for metabolites with large extracellular mass-fraction. We report that the co-precipitation is a hitherto neglected phenomenon and concluded that its degree strongly linked to culturing conditions (i.e. media composition) and chemical properties of the particular metabolite. Thus, intracellular metabolite levels measured from samples collected by cold methanol quenching might be uncertain and variably biased due to corruption by described phenomena.

Keywords Yeast · Sampling techniques · Quantitative metabolite measurements

Abbreviations

PCA	Perchloric acid
MES	2-(*N*-morpholino)ethanesulfonic acid
3PG	3-Phosphoglycerate [ChEBI:17794]
ADP	Adenosine diphosphate [ChEBI:16761]
aKG	2-Oxoglutarate [ChEBI:16810]
AMP	Adenosine monophosphate [ChEBI:16027]
ATP	Adenosine triphosphate [ChEBI:15422]
Cit	Citric acid [ChEBI:30769]
EtOH	Ethanol [ChEBI:16236]
F6P	Fructose-6-phosphate [ChEBI:16084]
Fum	Fumaric acid [ChEBI:18012]
G3P	Glycerone phosphate [ChEBI:16108]
G6P	Glucose-6-phosphate [ChEBI:17665]

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GC-IDMS	Gas-chromatography with isotope dilution mass spectrometry
GTP	Guanosine triphosphate [ChEBI:15996]
gDW	Gram of dry weight biomass
IS	Internal standard
Mal	Malic acid [ChEBI:30797]
MeOH	Methanol
PEP	Phosphoenolpyruvate [ChEBI:18021]
R5P	Ribose-5-phosphate [ChEBI:17797]
Suc	Succinic acid [ChEBI:15741]
UTP	Uridine triphosphate [ChEBI:15713]
T	Total content of metabolites in both extra- and intracellular fractions
EX	Extracellular fraction of metabolites
IN	Intracellular fraction of metabolites
QS	Quenching supernatant from cold methanol method
ME	Methanol extract from cold methanol method
P	Pellet from cold methanol method

1 Introduction

An accurate quantification of intracellular metabolites is a long-standing analytical challenge due to difficulties arising from metabolites' intrinsic low intracellular steady state concentrations, high turnover rate, instability of some (pH, temperature), leakage in course of a sampling and loss in course of multistep sample preparation. There were many attempts to develop a universal sampling/quenching/extraction method suitable for microorganisms to overcome mentioned critical points and deliver unbiased measurements of the intracellular metabolites. Canelas et al.(2008) have thoroughly compared different sampling, quenching and extraction methods in terms of the quantitative measurements of many different metabolites from yeasts and concluded that there is no consensus regarding effectiveness, reliability and reproducibility among these techniques. Recently, van Gulik et al. (2013) have concluded that the metabolism quenching is one of the most randomizing step among all others steps in sampling/quenching/extraction and there is no single condition suitable for quenching of different microorganisms, therefore it is necessary to validate and optimize overall process for each different microorganisms.

The variety of reported quenching methods can be classified into "sequential" and "simultaneous" operations or so-called workflows. The most popular sequential methods have been centered on the cold methanol quenching approach linked to different extraction techniques (Canelas et al. 2009; Gonzalez et al. 1997; Koning et al. 1992; Lange et al. 2001; Maharjan and Ferenci 2003). However, this method raises controversial discussions regarding theproblem of leakage of intracellular metabolites during quenching (Canelas et al. 2008; Loret et al. 2007; Mashego et al. 2007; Villas-Boas et al. 2005).

Originally, the method of cold methanol quenching was suggested by de Koning and Dam (Koning et al. 1992) as an alternative to classical simultaneous method of quenchingof metabolism and extraction of metabolitesby perchloric acid (PCA) (Reuss 1991; Theobald et al. 1997, 1993; Vaseghi et al. 1999). The authors have shown that quenching of yeast metabolism with cold methanol (mixing of 10–15 mL of sample with 60 mL of −40 °C 60 % aqueousmethanol) is equally fast and efficient and in addition hastwo advantages:(i) no leakage of intracellular metabolites, which allows a concentration step by separation of biomass carrying entrapped metabolites from the extracellular medium; (ii) the final neutral pH preserves acid-labile metabolites from decomposition.

However, later the metabolite leakage during cold methanol quenching was definitely provedin prokaryotes, while it was still arguable in eukaryotes. Particularly, it was explicitly shown that contact of bacterial cells with cold methanol solution (60 % v/v) results in leakage of intracellular metabolites from the cells into medium (Jensen et al. 1999; Letisse and Lindley 2000; Maharjan and Ferenci 2003; Wittmann et al. 2004). Therefore, it was concluded that metabolite leakage during cold methanol quenching is an organism specific phenomenon, which is related to the membrane composition and cell wall structure.

Further development in analysis of yeast intracellular metaboliteswas achieved by optimizingthe extraction step. Gonzalez et al.(1997) have suggested to combine the metabolism quenching by −40 °C 60 % buffered MeOH (5 mL sample + 26 mL of cold MeOH with 70 mM HEPES pH 7.5) with subsequent extraction of intracellular metabolites from biomass pellet by boiling 75 % buffered EtOH (with 0.25 M HEPES pH 7.5). After theseinvestigations the cold methanol quenching in combination with extraction by boiling ethanol has become popular in metabolomics research of yeasts (Hajjaj et al. 1998; Lange et al. 2001; Mashego et al. 2006, 2004; Visser et al. 2004; Wu et al. 2005). Later, it was additionally shown that quenching with cold aqueous methanol solution in combination withpure methanol extraction is also a reasonable compromise for global metabolomeanalysis in *Escherichia coli* (Maharjan and Ferenci 2003) and in *S. cerevisiae* (Villas-Boas et al. 2005).

In 2005 Villas-Boas et al. (2005) have presented a critical assessment of yeast samples quenched by different concentrations of cold aqueous methanol solutions and different extractions methods. The existence of metabolite

leakage phenomena in yeasts was quantitatively proved and it was addressed to loss of membrane integrity due to contact with methanol solutions. The degree of the leakage depends on both (i) the duration of contact and (ii) chemical properties of particular metabolites. Nevertheless, in some occasions the compromise was found, for example Loret et al. (2007) have claimed that only less than 5 % of intracellular metabolites leaked out during quenching at −40 °C with 60 % aqueous methanol solution.

Mashego et al.(2007) have reviewed all known quenching/extraction/analytical procedures for microbial metabolomics. They have concluded that there is no universal methodology in microbial metabolomics for instantaneous quenching of metabolic activity, extraction of all low molecular weight metabolites and analysis of metabolites of interest.The most challenging problem consists in the metabolite leakage during quenching, which is both organism and metabolite specific.

The problem of metabolite leakage can partly be overcome by applying simultaneous approaches where quenching and extraction are combined, such as perchloric acid-quenching/extraction (Theobald et al. 1993) or applying high temperature by injection of boiling water (Bhattacharya et al. 1995; Hiller et al. 2007; Hofmann et al. 2008) or passing the whole broth through a fast heat exchanger (Schaub and Reuss 2008; Schaub et al. 2006; Vielhauer et al. 2011). These methods allow measuring of the total mass of metabolites (T) in the fermentation broth. The simultaneous quenching/extraction methods require in any case an additional filtration step for independent analysis of the extracellular metabolites (EX) (Mashego et al. 2003; Taymaz-Nikerel et al. 2009; Vielhauer et al. 2011; Wittmann et al. 2004) in order to calculate the intracellular metabolite content ($IN = T - EX$).This is a differential approach which was outlined by Mashego et al.(2007) as the only adequate analytical solution to solve the leakage problem.The authors have also outlined that this approach should have certain disadvantages: metabolites are too diluted; sample matrix is complex and high salt content influences further analytical analysis. Thus, this approachwill work only with advanced and sensitive analytical methods like GC–MS or LC–MS, while the matrix effect is taken into account. Furthermore, they have concluded that it is more practical to develop techniques dedicated to targeting classes of metabolites according to their chemical properties. Additionally, the use of the differential sampling method was advocated in (Bolten et al. 2007)for sampling of microbial metabolites with medium-contained fraction (i.e. distributed both extra- and intracellularly). Nevertheless, van Gulik et al.(2013) have warned that high mass-fraction of extracellular metabolites can cause overestimation of intracellular metabolite levels, therefore leakage-free quenching method is essential for the quantitative metabolomics.

Later Canelas et al.(2008)have confirmed main observations made by Mashego et al. (2007).Particularly, they have analyzed awide range of metabolites of different properties ($n = 34$) in different sample fractions (whole-broth total; intracellular (from cell pellet); extracellular (filtrate); quenching solution; intracellular (calculated as $IN = T - EX$) and established metabolites' mass balancesto trace their fate during the quenching procedure. First of all, they have observed the metabolite leakage in course of the cold methanol quenching, and the leakage was higher for smaller and less polar metabolites.This conclusion is in line with (Bolten and Wittmann 2008), where only 5 % leakage of amino acids from yeasts was observed. Therefore, Canelas et al. (2008) have concluded that the extent of leakage depends on the exposure time (which is in agreement with Villas-Boas et al. (2005)) and the temperature and the properties of the methanol solution. Therefore, if these factors are not carefully treated, the conventional protocol of cold methanol quenching may result in considerable underestimation of the intracellular levels of most metabolites. As a solution, the authors have shown that the metabolite leakage can be entirely prevented by quenching with pure methanol (1:10; aq.sample/MeOH) at ≤−40 °C and minimized duration of the contact with the methanol solution (≤5 min). Additionally, it was clearly pointed out by the authors that in some cases medium components interfere with analytical techniques (e.g. high salt concentrations can cause peak-shifting in ion-exchange LC and ion-supression in MS) and therefore must be removed by washing step with ≥83 % methanol, although the washing step significantly contributes to the leakage.

The same authors (Canelas et al. 2009) have further re-evaluated different methods of metabolite extraction (hot water, boiling ethanol, chloroform methanol, freezing-thawing in methanol, acidic acetonitrile-methanol) regarding a wide range of intracellular metabolites ($n = 44$; phosphorylated intermediates, amino acids, organic acids, nucleotides). This investigation was performed under assumption of leakage-free quenching conditions found earlier in (Canelas et al. 2008). The authors have found that the choice of extraction method can drastically affect measured metabolite levels and the best performance, in terms of efficacy and metabolite recovery, were achieved with boiling ethanol and chloroform–methanol extraction methods.

Thus we can summarize that the degree of metabolite leakage in course of metabolism quenching by the cold methanol technique is a superposition of following contributing factors:

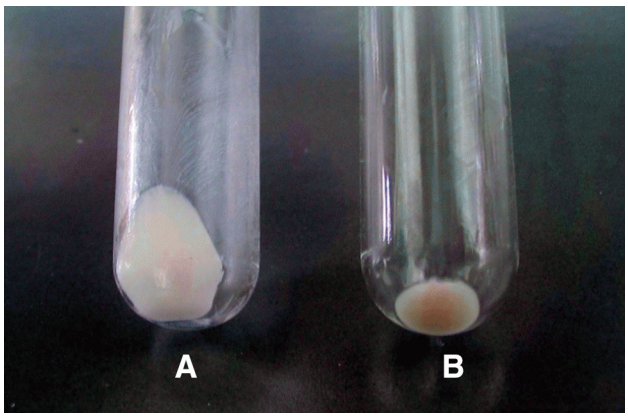

Fig. 1 Two identical samples of fermentation broth (**a** and **b**) of yeast growing on minimal mineral medium were identically quenched in cold (−40 °C) pure methanol in 5:1 ratio (MeOH:sample; v/v). After centrifugation and decantation of the methanol phase the sample **a** demonstrates considerable salt precipitates together with yeast biomass, whereas the sample **b** was additionally washed off with cold water resulting in salt-free yeast biomass

- Organism specific (i.e. prokaryotes, eukaryotes) membrane and cell wall compositions.
- Properties of methanol solution (e.g. final %).
- Duration of contact with methanol solution (≤5 min).
- Operation temperature (≤−40 °C).
- Chemical properties of metabolites.

Nevertheless, high reproducibility of the results obtained by the cold methanol quenching has brought van Gulik et al. (2013) to appropriateness of this method for the quantitative microbial metabolomics.

Therefore, the detailed description of the quenching and extraction procedures must be explicitly reported for each microbial and in vitro biology metabolomics experiments (van der Werf et al. 2007).

However, we think that yet another important factor influencing the metabolite quantification was overlooked in previous considerations, which shades and obscures the metabolite distribution among sample fractions during cold methanol quenching. Particularly, we have observed abundant precipitation of inorganic salts from minimal mineral growth medium during cold methanol quenching of yeast cultures (Fig. 1). This is not surprising, since salt solubility drastically decreases with increasing methanol content (Schellinger and Carr 2004).For example, potassium phosphate has a solubility of only about 20 mM in 80 % methanol at pH 7.0 and is almost insolublein ≥90 % methanol. Injection of the salt-containing broth sample (i.e. growth medium) into high concentration methanol solutions results in intensive flocculation. We will show that rapid salt precipitation might entrap dissolved metabolites and therefore contribute to the biasing of extracellular metabolites, whose pool can be additionally affected by the leakage. Therefore we have investigated the contribution of this phenomenon tometabolite mass distribution in the system (intra- and extracellular masses) during cold methanol quenching.

2 Materials and methods

The present work is a logical continuation of our previously published investigation on absolute quantification of metabolites using GS–IDMS (Vielhauer et al. 2011) and we employed the same experimental setup, conditions and sampling procedures.

2.1 Strain, medium and culturing conditions

The yeast *S. cerevisiae* haploid strain CEN.PK 113-7D[1] from glycerol stock was cultivated on agar in sealed plates at 30 °C and later some colonies were transferred into 5 mL liquid anaerobic CEN.PK medium prepared according to Verduyn et al. (1992) with 15 g/L of glucose and incubated overnight (up to OD_{660} ~0.1 OU). The preculture was inoculated into a steel KLF bioreactor (Bioengineering, Switzerland) and cultured in batch mode for 20 h, and only then switched to chemostat (weight controlled) mode. The growth conditions were: anaerobic CEN.PK medium (Verduyn et al. 1992) (see supplement for full composition of the medium), reactor volume 2 L, $D = 0.1\ h^{-1}$, glucose concentration in feed 50 g/L, pO_2 0 %, 30 °C, pH 5.0 ± 0.1 (adjusted by 3 M KOH), stirring 400 rpm, antifoam (1 mL per 3 h) and constant nitrogen sparging through the culture with 1.8 L/min and 0.1 bar headspace overpressure during cultivation and 0.7 bar during sampling. CO_2 in the off-gas was measured on-line by an Innova 1313 fermentation monitor (LumaSense Technologies, Denmark). After ~72 h the biomass in the chemostat has reached steady state at 3.4 gDW/L which corresponds to a biomass yield of $Y_{xs} = 0.07$ g biomass/g glucose. The steadiness of the culture was doubly checked through repeated measurement of dry biomass as well as by monitoring CO_2 production in off-gas. The culture was maintained in the chemostat no longer than 1 week to avoid adaptation processes (Mashego et al. 2005).

The final concentration of main salts in the anaerobic minimal growth medium was: $(NH)_2SO_4$ 15 g/L, $ZnSO_4$·$7H_2O$ 13.5 g/L, KH_2PO_4·9 g/L, $MgSO_4$·$7H_2O$ 1.5 g/L, $CuSO_4$·$5H_2O$ 0.9 g/L (see Supplement for full composition of the medium).

[1] The strain was kindly provided by Dr. Peter Kötter, Institute for Molecular Biosciences, Goethe University of Frankfurt, Germany.

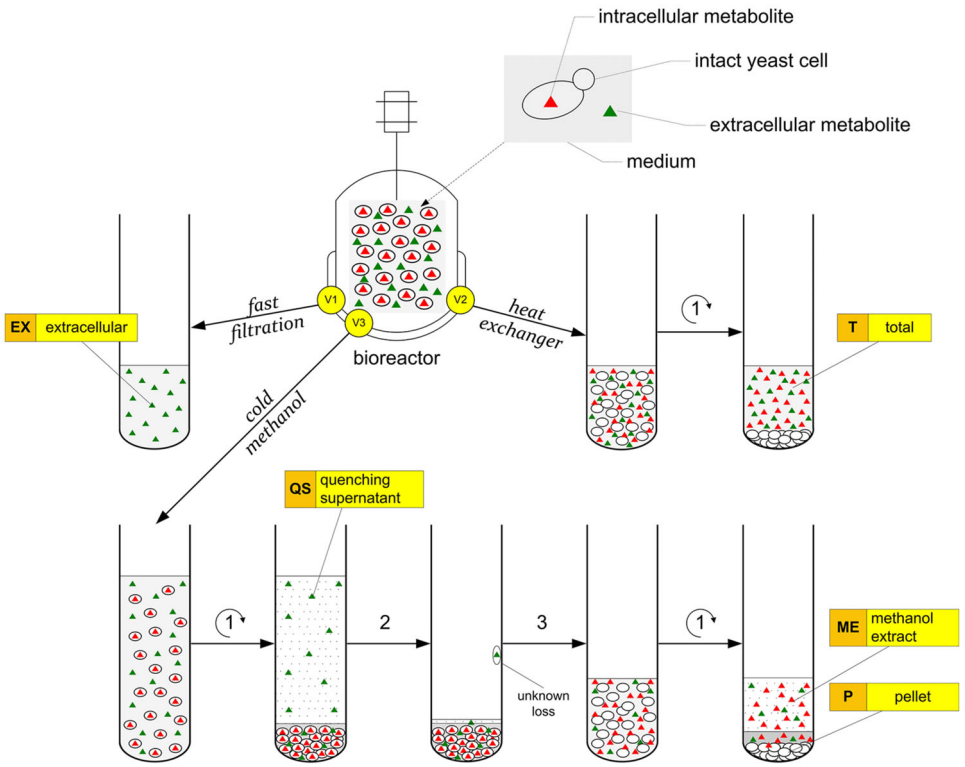

Fig. 2 The mass distribution of the extra- and intracellular metabolites was assessed by means of sampling the total mass of metabolites (*T*) through rapid heat exchanger; extracellular metabolites (*EX*) were sampled using fast filtration; whereas quenching supernatant (*QS*), methanol/water extracts (*ME*) and pellet (*P*) were sampled by conventional cold methanol quenching procedure. 1—centrifugation; 2—decantation of supernatant; 3—extraction with boiling 80 % methanol. There is unaccounted loss of metabolites due to unknown reasons in the sequential cold methanol method (unknown loss). V1, V2 and V3—sampling valves/ports. For the details see "Materials and methods" Section

2.2 Sampling

The total mass of the metabolites in the system (intra- and extracellular masses) was measured by two independent workflows: (i) differential method and (ii) conventional cold methanol quenching. According to Fig. 2, if a metabolite is distributed both extra- (*EX*) and intracellularly (*IN*), then its total mass (*T*) in the system (intra- and extracellular masses) should be comprised of:

$$T = EX + IN \quad \text{for differential method,} \tag{1}$$

$$T = QS + ME + P \quad \text{for conventional cold methanol,} \tag{2}$$

where *QS* is the quenching supernatant; *ME* is the methanol/water extract and *P* is the pellet fractions of the sample. Figure 2 may serve as the reference for easy identification of all analysed sample fractions.

The bioreactor had three sampling ports (Fig. 2) (Vielhauer et al. 2011): (V1) manual sampling for extracellular metabolites by fast filtering (Navon et al. 1979; Theobald et al. 1997, 1993); (V2) robotic sampling for total metabolites (from both biomass and medium) with aid of a fast heat exchanger at 97 °C (Schaub and Reuss 2008; Schaub et al. 2006); and (V3) robotic sampling for conventional 'cold methanol' quenching at −45 °C. The robotic samplers, control of chemostat conditions (dilution rate, gas pressure, etc.) during sampling procedure are described in details in (Vielhauer et al. 2011).

2.2.1 *Differential method*

2.2.1.1 T: total mass of metabolites (sampled with heat exchanger) For the sample notation please refer to Fig. 2. The 2.89 ± 0.08 mL samples of broth were collected through a single coiled steel tube (with 0.5 mm wall thickness and 2 mm inner diameter) submerged into a 97 °C thermostat (Schaub et al. 2006) and connected to a robotic sampler through V2 sampling port. The sampling flow rate was 3.6 mL/s, the residence time of the sample in the heat exchanger was 3.5 s and heating rate of the sample was ~135 °C/s (Schaub et al. 2006). The exact amount of each collected sample was determined gravimetrically. The collecting tray with sample tubes in the robotic sampler was filled with ice/water at 0 °C and it takes ~1 min to cool the sample to 0 °C after the heat exchanger. The withdrawn samples were additionally boiled at 95 °C for 5 min for complete extraction, then immediately cooled to 0 °C and centrifugated at 4 °C and 20 × 10^3 *g* for 5 min. Then the supernatant was collected, aliquoted and stored at −70 °C until further analysis. More detailed and depictured description of the sampling setup is in (Vielhauer et al. 2011).

2.2.1.2 EX: extracellular mass of metabolites (sampled with fast filtration) For the sample notation please refer to Fig. 2. Approximately 3 mL of the broth were quickly

sampled by over-pressure through sampling port 1 (manual valve V1) into a syringe (5 mL Braun® with Luer lock) containing ~3.85 g of pre-cooled (−20 °C) steel beads (4 mm, 15 beads per syringe). This method allows decreasing sample temperature down to ~0 °C in less than a second (Mashego et al. 2003; Theobald et al. 1997). The broth was immediately filtered through an attached syringe filter (Ratilabo®, CME, 0.45 µm, outer diameter 33 mm, with Luer lock, Carl Roche GmbH) directly into a pre-cooled (0 °C) tube and stored at −70 °C. For a skilled team the sampling procedure takes about 2 s per sample which corresponds to a sampling rate of ~1.5 mL/s. More detailed and depicted description of the sampling setup is in (Vielhauer et al. 2011).

2.2.2 *Cold methanol quenching method*

The samples were collected using a robotic sampler through the V3 sampling port (Fig. 2). The sampling system consisted of a three-position gate-valve V3 and the robotic sampler. The test tubes (polypropelene, 12 mL, Greiner, Germany) were filled with 9 mL of pure methanol (MeOH), weighed and pre-cooled to −40 °C. The collecting tray with sample tubes in the robotic sampler was filled with dry ice/EtOH at −45 °C according to (Canelas et al. 2008). The sampling system has an internal dead volume, which is usually filled with remained broth from the previous sampling. The entrapped biomass stagnates there and therefore must be discarded prior collecting the fresh sample to avoid its ingress into a test tube. Thus, the samples were withdrawn from a continuous outflow from the bioreactor. In the null-position V3 is opened in 1 → 3 direction towards waste, where the rate of outflow (4.6 mL/min) is controlled by peristaltic pump. At the designed sampling time-points, V3 switches to 1 → 2 position for t = 0.4 s to direct the broth flow into thetest tube and then returns to the 1 → 3 position. The broth was directly injected into cold pure methanol at −45 °C with flow rate of 3.6 mL/s (under P = 0.7 bar). The sample volume from the V3 port was 1.48 ± 0.03 mL. The final ratio of MeOH/sample achieved in the experiment was 6.1 ± 0.1; the final temperature of the mix was −35 ± 1 °C; the final methanol concentration of the mix was 86 %. Right after each sampling, the test tube was withdrawn from the robotic sampler, vortexed, weighed and placed in an external cryostat (Lauda, Germany) at −40 °C. It is important to note, that the extraction of the metabolites was performed directly after the sample collection. Using the cold methanol quenching method the following sample fractions were collected: quenching supernatant (QS), methanol extract (ME) and pellet (P) (for the notations see Fig. 2). More detailed description of the sampling setup and procedure is available in (Vielhauer et al. 2011).

2.2.2.1 QS: quenching supernatant The samples of 'quenching supernatant' were centrifuged at 4,000×*g* for 5 min at −20 °C. The supernatants (86 % methanol/14 % medium; v/v) were collected (fraction "QS") and stored at −70 °C until further analysis, whereas the tubes with remaining sediments were placed back in the cryostatat −40 °C.

2.2.2.2 ME and P fractions The pellets from 2.2.2.1, which consist of biomass together with precipitatedsalts,were taken from the cryostat at −40 °C and directly exposed to 98 °C in a boiling water bath for 1 min. Subsequently, the samples were immediately placed in ice water. 500 µl of 80 % methanol (v/v) and approx. 100 mg of pre-cleaned glass beads (1.2 mm) were added. The tubes were rigorously vortexed for 1 min in order to crush down the pellets, and the metabolites were extracted by incubation of sealed tubes for 2 min at 98 °C whilst shaking. After chilling the sample tube in ice water (0 °C), insoluble material was separated by centrifugation at 4,000×*g* for 10 min. The supernatants were stored at −70 °C until further analysis (fraction "ME").Additionally, the pellets were washed once with additional 500 µL of cold 80 % methanol and this methanol was merged with previous extracts. The washed pellets were re suspended in 1.5 mL of HPLC-grade water in order to re-dissolve precipitated salts and extract entrapped metabolites. Insoluble matter was removed by centrifugation at 4,000×*g* for 10 min, the supernatants were collected and stored at −70 °C until further analysis (fraction "P").

2.3 Metabolite analysis

2.3.1 *Metabolite analysis by GC–IDMS*

The gas chromatography (GC) analysis coupled with isotope dilution mass spectrometry (IDMS) was used for absolute metabolite quantification of extra- and intracellular metabolite levels and it was described previously in details (Vielhauer et al. 2011).

2.3.1.1 Preparation of U-^{13}C-labeled internal standard U-^{13}C-labeled lyophilized algal cells (>99 atom- % ^{13}C, lot no. 06741 EB, Isotec) were weighed into polypropylene tubes (screw cap, 12 mL; Greiner, Frickenhausen, Germany) in portions of 60–75 mg. The scale of the extractions was intentionally kept small in order to enable quick heating and cooling. About 5 mL of boiling water was added to each tube and the algal cells were extracted for 3 min at 95 °C whilst shaking. The tubes were chilled in ice-water, the resulting suspensions were pooled and centrifuged (0 °C, 4,000×*g*, 15 min). The clear supernatant was divided into 500-µl-aliquots and stored at −70 °C. Extracts from non-labeled algal cells (lot no. TV1530, Isotec) were prepared in the exact same manner.

2.3.1.2 Sample processing for GC-IDMS analysis To 40 µl of sample were added 50 µl of internal standard and 50 µl of *O*-methylhydroxylamine-hydrochloride (20 mg/mL in pyridine). The samples were evaporated to dryness in 1.5-mL Eppendorf tubes using a rotary vacuum concentrator (Speed-Dry 2-33IR, Christ, Osterode, Germany). Sample temperature was kept below 10 °C during evaporation by means of sample-specific drying programs (controlled drying-pressure and -temperature). Furthermore, residence time of dried samples in the concentrator was kept as short as possible (≤15 min). 60 µl of O-methylhydroxylamine-hydrochloride (20 mg/mL in pyridine) were added to the evaporated samples and the properly sealed tubes were incubated at 30 °C for 90 min during vigorous shaking. Insoluble matter was separated by centrifugation (r.t., 14,000×*g*, 5 min) and 40 µl of clear supernatant were transferred to a brown glass V-shaped micro-vial (0.8 mL, CS Chromatographie Service, Langerwehe, Germany) with screw cap and silicone/PTFE septa. After addition of 64 µl of *N, O*-bis(trimethylsilyl)trifluoroacetamide/trimethylchlorosilane (BSTFA/TMCS 99:1, Supelco), the vials were placed in a heating shaker (Eppendorf, Hamburg, Germany) equipped with an appropriate heater block suitable for standard autosampler vials (11.5 × 32.0 mm). Each well in the heater block was equipped with a dedicated PTFE support sleeve (Supelco) which ensured a proper position of the V-shaped vial. The silylation reaction was conducted at 50 °C with shaking for 60 min. The vials were cooled to r.t., vortexed and analyzed by GC–IDMS within 10 h.

Calibration standards were prepared from 50 µl of properly adjusted metabolite composition (solution in water), 50 µl of internal standard and 5 µl of *O*-methylhydroxylamine-hydrochloride (20 mg/mL in pyridine). The calibration standards were processed in the exact same manner as described above for samples.

2.3.1.3 GC–IDMS analysis In brief, GC–MS analysis was performed on Turbo Mass GC–MS system (PerkinElmer LAS, Rodgau, Germany) consisting of a Turbo Mass quadrupole mass selective detector coupled to an AutoSystem XL gas chromatograph. The mass-analyzer has been operated in the EI mode (70 eV), the ion source was heated to 180 °C and the transfer line to 280 °C. Measurements for quantification purposes were conducted using single ion monitoring (Toennessen, 2008). GC was performed on a Zebron ZB-5 ms column (30 m, 0.25 mm i.d., 0.25 µm film thickness; Phenomenx, Aschaffenburg, Germany) with helium as carrier gas at 1.0 mL/min (constant flow mode). Standard injection volume of the sample was 1 µl. The injector temperature was set to 230 °C and the sample transfer to the column was performed in the splitless mode. The temperature program was used: 5 min at 70 °C; 6 °C/min to 320 °C; 10 min at 320 °C. The GC–IDMS analysis and its validation were described previously in details (Vielhauer et al. 2011).

2.3.2 Nucleotides analysis by HPLC

Metabolites which could not be quantified via GC–MS (e.g.nucleotides) were measured by HPLC.

2.3.2.1 Sample preparation The solutions resulting from the integrated sampling procedure (fraction "T") and from filtrate sampling (fraction "EX"), respectively, were again clarified by centrifugation at 4 °C and 10,000 *g* for 15 min directly prior to analysis. 1,000 µl of fraction "QS" from the methanol quenching procedure were evaporated in a rotary vacuum concentrator (SpeedDry 2-33IR, Christ, Osterode, Germany) to a final volume of approx. 50 µl in order to remove methanol. The supernatants were diluted with 100 µl of water, centrifugated (4 °C, 10,000 *g*, 15 min) and analyzed by HPLC. 120 µl of fraction "ME" from the methanol quenching procedure were evaporated in the rotary vacuum concentrator to a final volume of approx. 30 µl in order to remove methanol. The supernatants were diluted with 150 µl of water, centrifugated (4 °C, 10,000 *g*, 15 min) and analyzed by HPLC. Aqueous fraction "P" from the methanol quenching procedure was centrifuged (4 °C, 10,000 *g*, 15 min) and the supernatant was analyzed by HPLC. As already mentioned, all evaporation and dilution steps were monitored gravimetrically.

2.3.2.2 HPLC analysis Nucleotides were measured using an HPLC system equipped with a binary high pressure gradient pump and a diode array detector (1200 Series; Agilent, Waldbronn, Germany). Reversed phase ion pairing HPLC was performed on a Supelcosil LC-18-T column (150 × 4.6 mm, 3 µm, Supelco) equipped with a Supelguard LC-18-T replacement cartridge (20 × 4.6 mm, Supelco) at a flow rate of 1.0 mL/min and 30 °C. Compounds were detected at signal wavelengths of 260 nm (reference 360 nm) and 340 nm (reference 450 nm), respectively. Peak assignment was confirmed by simultaneously recorded UV spectra (210–500 nm). The mobile phases were: (A) 0.1 M potassium phosphate buffer with 4 mM tetrabutylammonium bisulfate, pH 6.0, and (B) buffer A:methanol 70:30 (v/v), pH 7.2. The gradient program was used: 3.5 min at 0 % B; 1.818 % B/min from 0 to 30 % B; 2.5 % B/min from 30 to 35 % B; 1.389 % B/min from 35 to 60 % B; 5 % B/min from 60 to 100 % B; 7 min at 100 % B; 20 % B/min from 100 to 0 % B; 7 min at 0 % B.

2.4 Statistics

The standard error for calculated results was quantified according to the respective error propagation law. Unpaired *t* test was performed in GraphPad InStat (v3.06) with $P > 0.05$ significance level. ANOVA test of sample-to-sample variability in ME fraction was performed in GraphPad InStat (v3.06) with Bonferroni multiple comparisons post hoc test to compare all pairs of columns at $P > 0.05$ significance level.

Table 1 Specific metabolite content in studied fractions (Fig. 2) from yeast *S. cerevisiae* anaerobic continuous culture at D = 0.1 h^{-1} with 3.4 gDW/L. All values are in (μmol/gDW) ±SD (n—replicates)

		T	*EX*	*IN = T−EX*	*QS*	*ME*	*P*
GC–IDMS (μmol/gDW) ±SD (n)							
1	G6P	4.422 ± 0.311 (4)	0.397 ± 0.017 (4)	4.025 ± 0.312	0.17 ± 0.028 (2)	3.20 ± 0.238 (21)	0.425 ± 0.007 (2)
2	F6P	1.997 ± 0.117 (4)	0.19 ± 0.008 (4)	1.807 ± 0.117	0.08 ± 0.014 (2)	0.62 ± 0.054 (21)	0.065 ± 0.021 (2)
3	3PG	1.147 ± 0.095 (4)	0.615 ± 0.017 (4)	0.532 ± 0.097	0.18 ± 0.014 (2)	0.352 ± 0.025 (21)	0.515 ± 0.007 (2)
4	PEP	0.422 ± 0.045 (4)	0.045 ± 0.012 (4)	0.377 ± 0.047	n/d	0.132 ± 0.009 (21)	0.11 ± 0 (2)
5	R5P	1.295 ± 0.033 (4)	0.085 ± 0.005 (4)	1.21 ± 0.033	0.255 ± 0.049 (2)	0.628 ± 0.107 (21)	0.06 ± 0 (2)
6	G3P	4.797 ± 0.184 (4)	1.762 ± 0.036 (4)	3.035 ± 0.188	1.155 ± 0.077 (2)	2.224 ± 0.157 (21)	0.315 ± 0.021 (2)
7	aKG	10.885 ± 0.210 (4)	10.195 ± 0.081 (4)	0.69 ± 0.225	6.49 ± 0.056 (2)	1.220 ± 0.114 (21)	n/d
8	Suc	327.337 ± 6.245 (4)	319.8 ± 4.124 (4)	7.537 ± 7.484	409.79 ± 10.52 (2)	12.588 ± 1.497 (21)	0.92 ± 0.198(2)
9	Mal	26.195 ± 0.498 (4)	23.782 ± 0.242 (4)	2.412 ± 0.554	18.67 ± 0.169 (2)	5.163 ± 0.449 (21)	0.515 ± 0.007 (2)
10	Fum	2.12 ± 0.046 (4)	1.325 ± 0.021(4)	0.795 ± 0.051	1.35 ± 0.028 (2)	0.697 ± 0.047 (21)	n/d
11	Cit	40.355 ± 1.864 (4)	13.602 ± 0.788 (4)	26.752 ± 2.024	6.87 ± 0.042 (2)	15.604 ± 1.199 (21)	13.815 ± 0.445 (2)
HPLC (μmol/gDW) ±SD (*n*)							
12	AMP	1.48 ± 0.087 (4)	0.212 ± 0.019(4)	1.267 ± 0.089	0.185 ± 0.017 (4)	0.257 ± 0.067 (4)	1.448 ± 0.695 (6)
13	ADP	3.47 ± 0.093 (4)	0.225 ± 0.150 (4)	3.245 ± 0.177	n/d	1.725 ± 0.046 (4)	0.436 ± 0.086 (6)
14	ATP	3.997 ± 0.082 (4)	n/d	3.997 ± 0.082	n/d	4.462 ± 0.066 (4)	0.086 ± 0.013 (6)
15	GTP	0.46 ± 0.046 (4)	n/d	0.46 ± 0.047	n/d	0.592 ± 0.009 (4)	n/d
16	UTP	1.58 ± 0.092 (4)	n/d	1.58 ± 0.092	n/d	1.105 ± 0.013(4)	n/d

	QS + ME + P[a]	Intracellular by cold MeOH[b]	Entrapped in pellet[c]	Extraction efficiency[d]	Extracellular fraction[e]	Co-precipitation[f]	MSI identification level[g]
	% from *T*	% from IN	% from T	%	% from T	% from EX	
GC–IDMS (μmol/gDW) ± SD (n)							
1	86	80	10	88	9	57	1
2	38	34	3	91	10	58	1
3	91	66	45	41	54	71	1
4	57	35	26	55	11	100	1
5	73	52	5	91	7	−200	1
6	77	73	7	88	37	34	1
7	71	177	0	100	94	36	1
8	129	167	0	93	98	−28	1
9	93	214	2	91	91	21	1
10	97	88	0		63	−2	1
11	90	58	34	53	34	49	1

Table 1 continued

	$QS + ME + P$[a]	Intracellular by cold MeOH[b]	Entrapped in pellet[c]	Extraction efficiency[d]	Extracellular fraction[e]	Co-precipitation[f]	MSI identification level[g]
	% from T	% from IN	% from T	%	% from T	% from EX	
HPLC (μmol/gDW) ± SD (n)							
12	128	20	98	15	14	13	1
13	62	53	13	80	6	100	1
14	114	112	2	98	0		1
15	129	129	0	100	0		1
16	70	70	0	100	0		1

Negative values might indicate leakage

Metabolite amounts in T total broth measured by differential method, $QS + ME + P$ total amount of metabolite measured by cold methanol method, *EX* extracellular, *IN* intracellular, *QS* quenching supernatant, *ME* methanol extract, *P* pellet (graphical notations are at Fig. 2), *n/d* not detected. All the abbreviation of the metabolites and methods are in accordance with the abbreviation list

[a] Total fraction of metabolite collected by cold methanol procedure relative to differential method ($100 \times (QS + ME + P)/T$)

[b] Intracellular fraction of metabolite measured by cold methanol procedure relative to differential method ($100 \times ME/(T-EX)$)

[c] Fraction of metabolite entrapped in salt pellet ($100 \times P/T$)

[d] Extraction efficiency of entrapped metabolite from pellet ($100 \times ME/(ME + P)$)

[e] Fraction of metabolite presented extracellularly in the system ($100 \times EX/T$)

[f] Fraction of extracellular metabolite that has co-precipitated with salts into pellet ($100 \times (EX-QS)/EX$)

[g] The metabolomics standards initiative (MSI) identification levels according to Sumner et al. (2007)

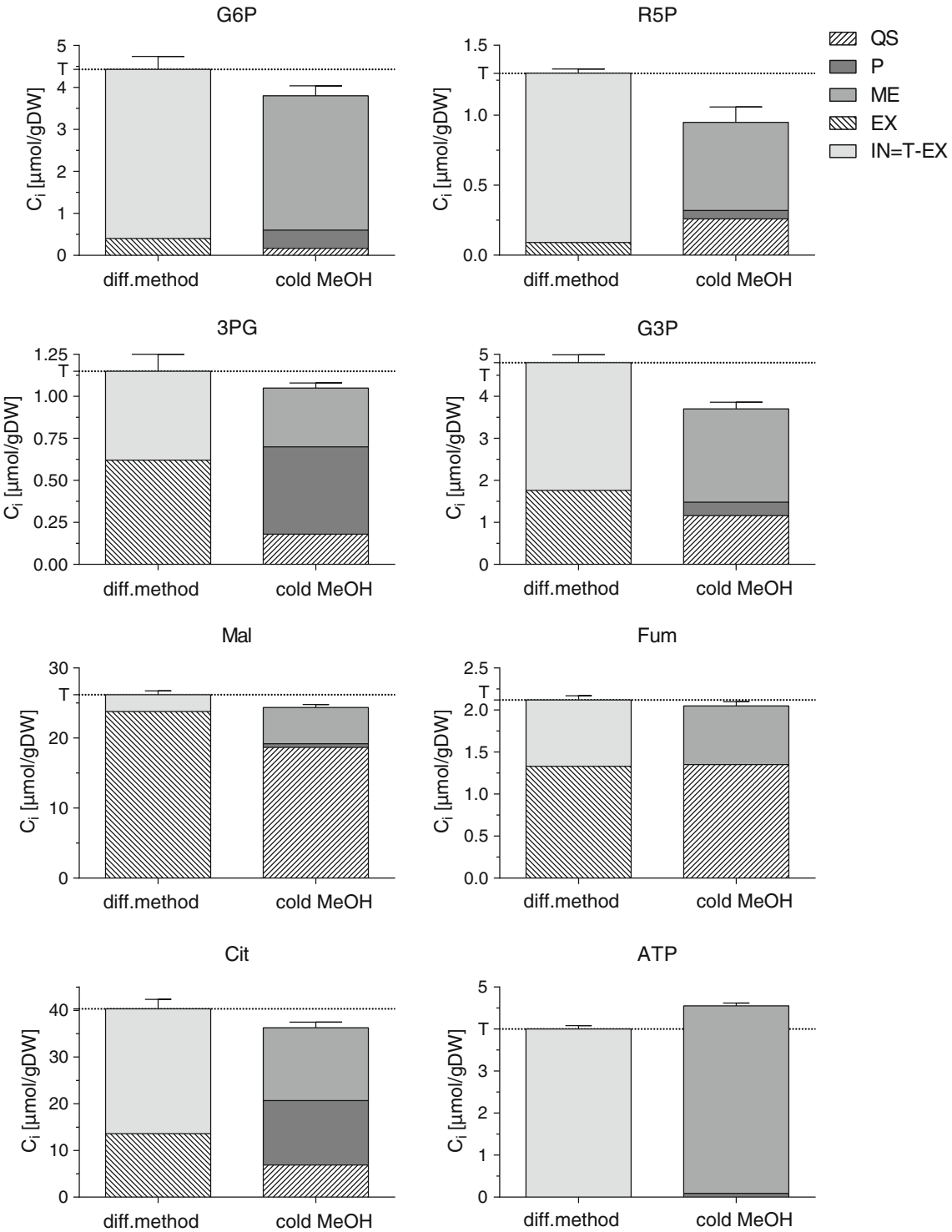

Fig. 3 Full mass balance of selected (from Table 1) metabolites in different sample fractions obtained by different sampling techniques from the same anaerobic continuous glucose limited yeast *S. cerevisiae* CEN.PK 113-7D culture at D = 0.1 h^{-1} and 3.4 gDW/L. For numerical data refer to Table 1; for fraction notations refer to Fig. 2; for metabolite notations refer to the abbreviation list. Where: *diff. method*—metabolite quantification by the differential method; *cold MeOH*—metabolite quantification by the cold methanol method; *T* and *dotted line* represent total amount of metabolite measured by the differential method

3 Results and discussions

Main results of metabolite quantification in all experimental fractions are summarized in Table 1. Using GC–IDMS and HPLC analytical methods overall we have quantified 16 metabolites in five distinct fractions (Fig. 2). Based on our previous findings (Vielhauer et al. 2011), the differential method is considered as thebenchmark in absolute quantification of the intracellular metabolite to judge an efficiency of the cold methanol quenching method in this research.

3.1 Systematic bias of metabolites during cold methanol procedure

A systematic bias of the majority of the metabolites during the cold methanol workflow was revealed from the comparison of total amounts of metabolites collected by the differential method (as *T*) with the total amount of metabolites collected by the cold methanol workflow (*QS* + *ME* + *P*) (Fig. 3). The Table 1 shows the degree of metabolite recovery by cold methanol workflow as 100 × [(*QS* + *ME* + *P*)/*T*]. Bearing in mind, that the differential method and cold methanol quenching represent completely independent workflows, the mass balance closure seems to be very reasonable within the error of measurement (on average 83 %).

We assume several reasons for the metabolite loss in course of conventional cold methanol quenching workflow:

i. Multistep unit operations result in unavoidable loss of metabolites, which might appear during removal of the quenching supernatant (brittle pellet),
ii. Thermal decomposition during heat shock inactivation,
iii. Incomplete pellet extraction,
iv. Chemical decomposition during the time-consuming overall procedure.

A particularly critical point is the heat inactivation of metabolism prior to metabolite extraction. The temperature shift from −40 °C to >90 °C has to be conducted very quickly (sub-second range!). Otherwise some enzymatic activities could be re-started partially for a limited period of time. In contrast to the employed heat exchanger, the heat inactivation performed by boiling solvent is not thoroughly characterized concerning speed and metabolite recovery (Gonzalez et al. 1997; Schaub et al. 2006). Furthermore, its distinctly manual character may adversely influence the sample-to-sample reproducibility. We observed higher metabolite levels when performing an additional "dry" heat inactivation step (see experimental).

3.2 Metabolite co-precipitation with salts

The metabolite amount in the *QS* fraction from the cold methanol workflow is supposed to be equivalent to the *EX* fraction from the differential workflow (Fig. 2). However, there is significant mismatch (either loss or gain) between them (Table 1). Therefore, we hypothesize two major reasons that can contribute to this mismatch:

i. Metabolite leakage (should result in gain of metabolites in the QS fraction)
ii. Metabolite co-precipitation with inorganic salts (should result in loss of metabolites from the QS fraction)

Leakage of intracellular metabolites can only be proved by an increased content of metabolites in the *QS* fraction compared to the *EX* fraction. In fact, we observed indication for the leakage only for succinate and R5P, while all the rest intracellular metabolites had lower concentration in the *QS* fraction than in the *EX* fraction (Table 1). From the other side, since we have observed metabolites that were entrapped in the precipitated salts (column 'entrapped in pellet' at Table 1), than we can assume that the loss of the metabolites from the *QS* fraction can be explained by co-precipitation with inorganic salts. Consequently, if an intracellular metabolite has been simultaneously subjected to the leakage and co-precipitation with flocculating salts during the cold methanol quenching, then we cannot segregate particular contribution of these both phenomena from our experimental design in gain and loss of that metabolite in the *QS* fraction, but only can report the final outcome. However, loss of the overall amounts of the most metabolites in the *QS* fraction and their appearance in the salt pellet indicate a superiority of the co-precipitation phenomenon over the leakage phenomenon for the most metabolites in balance of the metabolite amounts in the *QS* fraction.

3.2.1 Problems caused by precipitated salts

It is well-known, that several water-soluble inorganic salts are poorly soluble in methanol. Available data on the solubility of commonly used phosphate salts in aqueous methanol demonstrate, that solubility declines dramatically with increasing methanol content (Schellinger and Carr 2004). According to the recommendations for leakage-free quenching (Canelas et al. 2008), the final methanol concentration should be ≥83 % (v/v) which corresponds to solubility of only 13 mM of potassium phosphate at pH 7 and 25 °C (Schellinger and Carr 2004). As the solubility is likely to be considerably lower at −40 °C, we carried out own measurements and estimated a solubility of 3 mM of

potassium phosphate (pH 7) in 85 % methanol (v/v) at −40 °C. Based on the experimental conditions used in the present work (culture medium contains ~66 mM monopotassium phosphate, final methanol concentration after quenching is 86 %), this will result in the precipitation of about 95 % of the potassium phosphate salt. Other inorganic salt components in the medium (primarily sulfates) may display a similar behaviour. During our experiments we could clearly observe distinct salt precipitates in the methanol quenching pellet, which could be easily redissolved in water (Fig. 1).

Pelleted cells are frequently disrupted using boiling ethanol, which later is evaporated and the resulting pellet is re-suspended in water (Canelas et al. 2008). However, depending on used ethanol concentrations this can even enrich inorganic salts precipitate.

High inorganic salt contents in the samples frequently cause problems with analytical procedures: ion-suppression (LC–MS) or interference with sample derivatization (GC–MS). Thus, in order to minimize salt transfer to the samples for GC–MS, we have used 80 % methanol for metabolite extraction instead of the pure water. This approach allows keeping most of inorganic salts undissolved (for comparison of different extraction techniques see (Canelas et al. 2009)) while extracting entrapped metabolites form the salt pellet.

Apart from troublesome effect to analytical procedures, samples and analyses, the precipitated salts cause significant overestimation of some metabolites (i.e. Suc, Mal and aKG; Table 1) by the methanol quenching procedure. The extracellular mass-fraction of these metabolites is >90 % (see Table 1). Thus, incomplete removal of the extracellular metabolite portion seems to account for incorrect results. But especially in the case of Mal, an overestimation of more than 100 % at an extracellular fraction of about 90 % can hardly be explained by insufficient removal of the supernatant. In fact, there seems to be some kind of "enrichment" in the pellet. This could be an indication of a co-precipitation phenomenon. Co-precipitation can be estimated from the fractions *QS* and *EX* as a relative amount of lost metabolites from the extracellular compartment ($100 \times (EX-QS)/EX$; Table 1). The Table 1 reveals, that actually considerable co-precipitation occurs with most of the measured metabolites. Phosphorylated, and hence the most polar compounds, are most notably affected. Only Suc and Fum, comparatively less polar species, make an exception. Fum seems to be the only metabolite exhibiting "ideal" behaviour with respect to comparability of methanol quenching and differential method.

On the other hand, co-precipitation does not necessarily lead to overestimation. Most metabolites are underestimated in the *ME* fraction, in spite of carry-over from the extracellular medium into the pellet. Apparently, another effect has to be considered. Incomplete extraction of metabolites from the pellet with boiling 80 % methanol might be a self-evident explanation. The extraction efficiency of the entrapped metabolites from the pellet can be derived from fractions *ME* and *P* ($100 \times ME/(ME + P)$; Table 1). Data from Table 1 demonstrate, that highly polar metabolites are not entirely extracted by 80 % methanol. Above all, 3PG, Cit and PEP "stick" heavily to the pellet. Thus, our attempt to extract metabolites from the pellet without re-dissolving the major amount of salts didn't succeed. The only way to completely extract metabolites seems to be the dissolution of precipitated salts using water as the extraction solvent, resulting in an unfavourable enrichment of inorganic salts in the analytical sample.

3.3 Misestimation of intracellular metabolite content

The intracellular metabolites are extracted in both workflows as the *ME* fraction in the cold methanol workflow and the *IN* fraction in the differential workflow and their concentrations are supposed to be equivalent in both these fractions (Fig. 2).If the cold methanol quenching is assumed to be leakage free (Canelas et al. 2008), then metabolite contents in the *ME* fraction from the cold methanol quenching procedure should be equal to *IN* from the differential method. However, these quantities do not match in all instances (Table 1; Fig. 3). If *IN* from the differential method is used as a benchmark, then in most cases the intracellular content (*ME*) of metabolites is underestimated by the cold methanol quenching, except for oxoglutaric (aKG), malic and succinic acids which are overestimated.

We have hypothesized that misestimation of the intracellular metabolite contents by the cold methanol method might be explained by following possible reasons:

i. Metabolite leakage in course of the quenching
ii. Incomplete removal of supernatant from the pellet
iii. Partial solubilization of metabolites which were co-precipitated in the salt pellet

Two last points are especially significant for metabolites with high extracellular mass fraction.

3.3.1 Metabolite leakage

Although, as we have stated, we cannot directly estimate metabolite leakage from our experimental design, but the mismatch between metabolite amounts in the *ME* and *IN* fractions can be attributed to the possibility of metabolite leakage from cell to medium in course of the cold methanol quenching. We have quantified the relative content of intracellular metabolites measured from the *ME* fraction

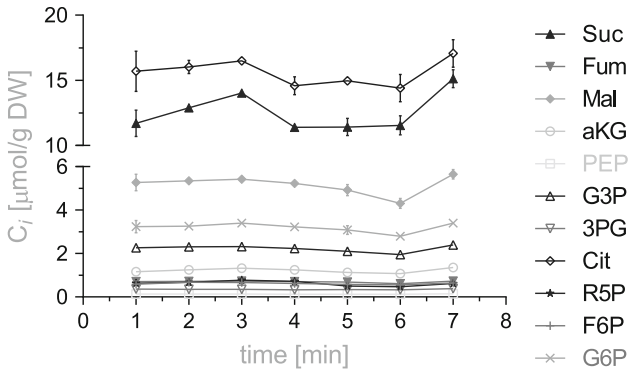

Fig. 4 Repeated measure of steady state concentrations (±SD, $n = 3$ per sample) of intracellular metabolites in *ME* fraction quantified by GC–IDMS in yeast *S. cerevisiae* growing anaerobically in chemostat at $D = 0.1\ h^{-1}$. Samples were collected using automated robotized sampling setup. ANOVA with Bonferroni multiple comparisons post hoc test has revealed significant sample-to-sample variability for all metabolites at $P < 0.001$

relative to the *IN* fraction as *ME/(T−EX)* (column: intracellular by cold MeOH; Table 1). This data indicate % of missed or gained metabolites in intracellular compartment in course of the cold methanol workflow. For some metabolites (like PEP, F6P) the loss is more than 50 % resulting in significant underestimation of their concentrations by cold methanol method. Interestingly, some organic acids (αKG, malic and succinic) have demonstrated opposite trend—gaining content! It is likely that this can be attributed to their large extracellular concentration and related analytical difficulties.

3.3.2 *Incomplete removal of the supernatant*

The unavoidably incomplete removal of the quenching supernatant from the cell/salt pellet leads to an overestimation of intracellular concentrations (no washing step was conducted according to (Canelas et al. 2008) in order to avoid the leakage). Some metabolites are presented in very large mass fraction in the extracellular compartment relative to the intracellular mass fraction (column: extracellular fraction % from *T*; Table 1). Therefore minor residues of the quenching supernatant adhered to cell/salt pellet or walls of the test-tube might lead to the significant overestimation of the intracellular levels of these metabolites. In fact, this can explain overestimation of αKG, Suc and Mal by the cold methanol workflow (column: intracellular by cold MeOH % from *IN*; Table 1).

3.3.3 *Solubilization of metabolites precipitated in the salt pellet*

Typically, there are three sequential steps to extract metabolites from the cell pellet collected by the cold methanol quenching (Canelas et al. 2008): (i) cell disruption by boiling with 80 % ethanol; (ii) evaporation to dryness; (iii) extraction of metabolites by water. The last step results in dissolution of pelleted inorganic salts that have precipitated in course of quenching with pure cold methanol. Thus, to avoid salt penetration to the GC-samples we have tried to reduce this procedure down to a single step by using 80 % methanol in steps (i) and (iii). Correspondingly, this reduces the whole procedure to a single step, i.e. simultaneous cell disruption and metabolite extraction with 80 % boiling methanol. The advantages of 80 % boiling methanol are: (i) quenches remained metabolic activity; (ii) disrupts yeast cells; while (iii) not re-dissolving the entire mass of inorganic salts.

Unfortunately, we have observed incomplete extraction of metabolites from the pellet by using 80 % methanol (extraction efficiency $100 \times ME/(ME + P)$; Table 1) and the remained portion of the metabolite could only be extracted using water as a solvent (fraction *P*; Table 1). Consequently, the classical extraction of metabolites from precipitated cells with water (step iii) will inevitably result in carry-over of entrapped metabolites from the precipitated salts to the *ME* fraction and correspondingly significantly contribute to the misestimation of the intracellular metabolite concentrations.

3.3.4 *High extracellular concentrations*

Almost all studied intracellular metabolites from central carbon metabolism (e.g. carboxylic acids, phosphorylated sugars, phosphorylated glycerides and nucleotides) were present extracellularly in relevant amounts. Some intracellular metabolites show a very high extracellular mass-fraction ($100 \times EX/T$; Table 1), thus their major mass is extracellular. As a consequence, their calculated $IN = T-EX$ values exhibit a large relative error (Table 1), which points to the low reliability of the measurements for this particular metabolites by the differential method employing GC–IDMS. For example, succinate was present at extracellular concentrations that were too high to allow accurate intracellular determinations by GC–IDMS via the differential method (Table 1) (Vielhauer et al. 2011). On the other hand, the concentrations were far beyond a useful calibration range of the GC–IDMS method (Vielhauer et al. 2011). Additionally, the differential approach possesses the intrinsic drawback of being unable to predict accurate intracellular data if the extracellular fraction is too high (Taymaz-Nikerel et al. 2009). If the mass fraction of an extracellular pool of metabolite is more than 90 %, the relative error of the calculated intracellular metabolite content ($IN = T-EX$) may easily exceed 20 % (Vielhauer et al. 2011). Alternatively, extracellular data for metabolites present in such high extracellular concentrations could

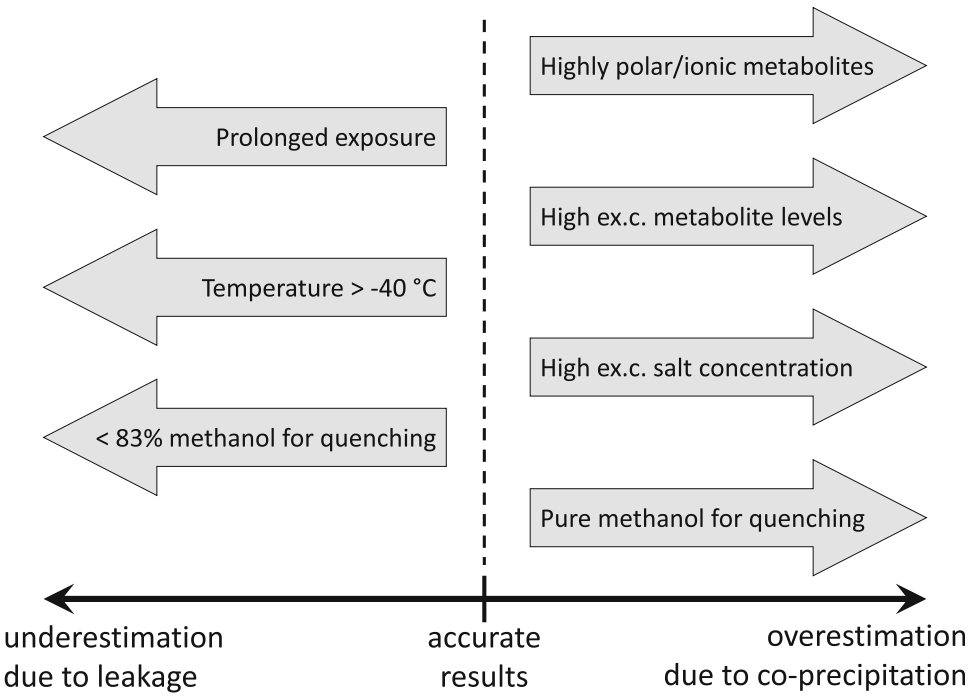

Fig. 5 Generalized conclusions on the "cold methanol quenching": Intracellular metabolite levels deriving from cold methanol quenching procedures is corrupted by leakage and co-precipitation phenomena. This phenomenon strongly depends on composition of fermentation medium. Application-specific validation of any sampling/quenching/extraction procedure seems to be generally inevitable. Where: *ex.c.*—extracellular

be accurately measured using other analytical methods instead.

3.4 Reproducibility of intracellular metabolite quantification obtained by cold methanol workflow

The repeated sampling ($m = 7$, once per minute) of intracellular metabolites was performed by conventional cold methanol quenching (Fig. 4) and each *ME* fraction was quantified by GC–IDMS (Vielhauer et al. 2011) in triplicates ($n = 3$). All time-points were sampled from the same steady state culture therefore we assume that the data are sampled from population that follows Gaussian distribution and therefore should have identical SDs. The Kolmogorov–Smirnov normality test of pooled dataset has shown that the data passed the normality test with $P > 0.05$. The sample-to-sample variability for all metabolites was analyzed by ANOVA with Tukey–Kramer multiple comparisons post hoc test has revealed that the P value is <0.01 for all metabolites, which means that variation among column means is significantly greater than expected by chance. Additional ANOVA post hoc test for linear trend between column means and column number has not revealed any statistically significant linear trend (at $P > 0.1$) whereas the remaining variation amongcolumn means is significant (at $P < 0.01$).

Thus, despite statistically significant sample-to-sample variation along the sampling time, the linear trend has zero-slope, which proves that the observed variability is the result of the error randomization. We assume that one of the reasons for sample-to-sample variation in the cold methanol workflow might be related to randomization of loss/gain of metabolites due to unaccounted phenomena, e.g. co-precipitation, decomposition and metabolite loss during multistep sample preparation and etc. Additionally, we conclude that if the aqueous solvents (which allow dissolution of the pelleted salts) will be used for the metabolite extraction from the collected biomass pellet, than inevitable uncontrolled solubilization of the co-precipitated extracellular metabolites from the pellet will take place which will bias the measurements of the intracellular metabolite content.

3.5 Considerations on quenching with pure methanol

The processes occurring during the quenching procedure using pure methanol are quite complex. Figure 2 visualizes the different steps of the sampling operations. Most of the inorganic salts from the culture medium precipitate upon injection of the broth into high % aqueous methanol and extracellular metabolites are co-precipitated in different extent with flocculating salts. The separation of the supernatant from the pellet is usually incomplete due to retaining of the small part of the supernatant within the pellet. This introduces bias to metabolites with high extracellular mass fraction. Cell disruption/heat inactivation using boiling solvent or a "dry" heat shock is a delicate step, because heat-up time cannot reliably be controlled. Metabolite extraction will either be incomplete (extracted with organic solvents) or result in very high salt

loads (extracted with water). The high number of manual unit operations decreases the sample-to-sample reproducibility.

All mentioned aspects will influence the recovery of a certain metabolite. Table 1 lists quantitative data for extracellular fraction, co-precipitation, extraction efficiency and overall recovery of 16 metabolites. Obviously, each metabolite displays an individual behavior pattern with regard to the different steps of the procedure. The experimentally observed recovery is thus a result of the interplay between co-precipitation (increasing effect) and incomplete extraction (decreasing effect). It is dependent on culture conditions (extracellular metabolite levels), medium composition (salt contents), the thermal and chemical stability of the metabolite as well as its polarity. Most of these difficulties might be overcome by adding internal standards at the earliest stage possible, i.e. after removal of the quenching supernatant, but to do so would not circumvent the co-precipitation problem. Thus, we have summarized and generalized all discussed contributions to the metabolite misestimation due to salt precipitation and leakage in course of cold methanol sampling in Fig. 5.

4 Concluding remarks

By application of the aforementioned GC–IDMS methodology to the measurement of intracellular metabolite levels in anaerobic chemostat cultures of the yeast *S. cerevisiae* CEN.PK 113-7D, co-precipitation of extracellular metabolites during quenching with pure methanol at −40 °C could be demonstrated. However, our data cannot segregate the contribution of this phenomenon from the metabolite leakage in mass balance of the extracellular metabolites of the collected samples. The bias caused by this phenomenon mainly connected to the composition of the fermentation media and on the current extracellular metabolite levels. In summary, the accuracy of metabolite levels deriving from cold methanol quenching is dependent on current fermentation conditions. We have observed that in our case more reproducible estimates of the intracellular (*IN*) content of most metabolites could be achieved by the differential approach. The approach calculates difference between total (*T*) (acquired from a fast heat exchanger-based integrated sampling device) and extracellular (*EX*) (acquired from fast filtration) metabolite contents ($IN = T - EX$). We have to admit that this method has some uncertainties in quantification of intracellular metabolite concentrations that have high extracellular concentration and mass-fraction. We are currently applying this strategy for dynamic analysis of intracellular phosphorylated metabolites with low extracellular fraction in course of glucose pulse experiments on anaerobic *S. cerevisiae* continuous culture.

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