Analytical Biochemistry 394 (2009) 192–201

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

A method for enzyme quenching in microbial metabolome analysis successfully applied to gram-positive and gram-negative bacteria and yeast

Jana Spura, Lorenz Christian Reimer, Patricia Wieloch, Kerstin Schreiber, Sebastian Buchinger [1], Dietmar Schomburg *

Department of Bioinformatics and Biochemistry, Technische Universität Braunschweig, 38106 Braunschweig, Germany

ARTICLE INFO

Article history:
Received 16 March 2009
Available online 15 July 2009

Keywords:
Metabolome analysis
Quenching
Escherichia coli
Corynebacterium glutamicum
Saccharomyces cerevisiae

ABSTRACT

Although microbial metabolome analysis has now become a widely used method, no generally applicable quenching method has been published so far. Either the methods were established for only one defined organism or the metabolite coverage was quite low. In the current work, a novel, reliable, and robust quenching method for different types of organisms is described. Compared with the commonly used quenching procedure with 60% methanol (−50 °C), we obtained improved results for three examined organisms with different cell wall and membrane structures using a 40% ethanol/0.8% sodium chloride solution (−20 °C). Increased metabolite levels were achieved for 60–80% of all identified compounds. Moreover, the estimated standard error of the relative concentrations of 120–160 different substances was only 14 ± 4% compared with 17 ± 3% in unquenched samples and 24 ± 7% in samples quenched with methanol for the different tested organisms.

Since the development of applicable methods for microbial metabolome analysis using different techniques such as nuclear magnetic resonance (NMR)[2] spectroscopy and mass spectrometry following a separation step [1–3], the accurate quantitative analysis of intracellular metabolite pools has became a widely used analytical technique for cellular characterization. The different analytical techniques all share one critical step: sampling followed by the separation of cells and medium. Many intracellular metabolites are characterized by a rapid turnover and sometimes low pool sizes. Thus, metabolite levels can change significantly during cell harvesting. To circumvent this problem, the cellular metabolism must be stopped as quickly as possible, a process often referred to as quenching. Recently, immediate harvesting without separation of intra- and extracellular metabolites or medium was suggested [4]. In this case, cell leakage is no concern, but the differentiation and interpretation of metabolite levels must be done very carefully to circumvent overestimation [5]. In addition, components of the medium might cause problems during analysis, in particular those with a high concentration.

So, for an exact determination of endo- and exometabolome, a reliable quenching method is essential. A number of different methods have been developed for quenching of microbial metabolism. Rapid changes in temperature or pH are mostly performed. Often organic solvents are used.

For the eukaryotic organism *Saccharomyces cerevisiae,* a common but often controversially discussed method is quenching with a solution of 60% methanol at approximately −50 °C [6–10]. However, when using this procedure for prokaryotic microbes, leakage of the cells was observed in some cases, the so-called "cold shock" phenomenon [11]. Nevertheless, this method still seems to be the one applied most often [5,12,13]. Organism-dependent variations are often done to improve results of methanol quenching, including different temperatures of the quenching solution, different concentrations of the quenching agent, and different buffers [14–18]. Other approaches using liquid nitrogen [10,19,20], perchloric acid [21–23], boiling ethanol [10,12,24], and cold ethanol [24], as well as cold pure methanol [4], a cold glycerol–saline solution [25], and hot sodium hydroxide [23], have been published. However, most of these methods are tested and optimized for only one organism or result in a very small number of detectable metabolites. A satisfying approach for different types of organisms—gram-negative prokaryotes, gram-positive prokaryotes, and eukaryotes—is still missing. The requirements for such a quenching method are (i) robustness and reliability, (ii) prevention of cell leakage, (iii) a fast procedure that can be applied for routine work, and (iv) the generation of reproducible results for a high number of metabolites.

This article describes a new quenching method fulfilling these criteria to a large extent. The quenching solution is composed of

* Corresponding author. Fax: +49 531 3918302.
E-mail address: d.schomburg@tu-bs.de (D. Schomburg).

[1] Present address: Department of Ecotoxicology and Biochemistry, Federal Institute of Hydrology, 56068 Koblenz, Germany.

[2] *Abbreviations used:* NMR, nuclear magnetic resonance; MSTFA, *N*-methyl-*N*-trimethylsilyltrifluoroacetamide; PTV, programmed temperature vaporizer; MCF, methyl chloroformate.

0003-2697/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2009.07.016

40% ethanol and 0.8% (w/v) sodium chloride at −20 °C. The used alcoholic compound and its concentration, the amount of salt, and the applied temperature during quenching were optimized, representing the most important parameters.

Ethanol was used instead of methanol, and the concentration of alcohol was reduced to prevent damage of the cell membrane before cell extraction. Both alcohols can affect the integrity of the membrane. However, previous publications mentioned different observations for methanol, stating that pure methanol could lead to good results [4].

Our performance was developed and tested on three different organisms: *Saccharomyces cerevisiae* (eukaryote), *Corynebacterium glutamicum* (gram-positive prokaryote), and *Escherichia coli* (gram-negative prokaryote). Results were compared with a published standard method [2,26] as a nonquenched basis and with the currently most widely applied quenching method using cold methanol solution [6–8]. The cold glycerol–saline method [25] was tested as well.

Materials and methods

Chemicals

MSTFA (*N*-methyl-*N*-trimethylsilyltrifluoroacetamide) used for derivatization was purchased from CS Chromatography Service (Langerwehe, Germany), pyridine was purchased from Fluka (Munich, Germany), and methoxyamine hydrochloride was purchased from Sigma–Aldrich Chemie (Munich, Germany).

Microbial cultivations, media, and sampling

All cultivations were done according to standardized reproducible protocols performed routinely in our lab using minimal media. Organisms were cultivated in shaking flasks as batch cultures according to their specific requirements. Cells were always grown until the middle exponential growth phase to ensure a defined metabolic state. Nevertheless, to minimize differences due to changes in substrate concentration and oxygen supply during cultivation, unquenched samples were always treated in parallel with the quenched sample set. Therefore, to compare the different quenching methods, samples were always taken in parallel from the same cultivation and at least in triplicates.

The effect of quenching for each organism was examined in three independent experiments.

C. glutamicum

The *C. glutamicum* wild-type strain ATCC 13032 was purchased from the American Type Culture Collection (Rockville, MD, USA). After cultivation of bacteria in 37 g/L brain heart infusion medium (Difco, Detroit, MI, USA) at 30 °C for 7 h on a rotary shaker at 180 rpm, 1 ml of this culture was transferred into 50 ml of minimal medium and incubated for approximately 16 h at 30 °C and 180 rpm. For the main culture, cells were resuspended in 100 ml of minimal medium to an optical density at 600 nm (OD_{600}) of 1 and grown until an OD_{600} of 10. For metabolome analysis, 5 ml was taken for each sample. Here 1 L of the minimal medium (pH 7.0) contained 5 g of $(NH_4)_2SO_4$, 5 g of urea, 2 g of $K_2HPO_4 * 3H_2O$, 2 g of KH_2PO_4, 0.25 g of $MgSO_4 * 7\ H_2O$, 0.01 g of $CaCl_2$, 0.2 mg of biotin, 20 g of glucose, 28.5 mg of $FeSO_4 * 7\ H_2O$, 16.5 mg of $MnSO_4 * H_2O$, 6.4 mg of $ZnSO_4 * 7\ H_2O$, 0.764 mg of $CuSO_4 * 5\ H_2O$, and 0.44 mg of $NiCl * 6\ H_2O$ (modified after Ref. [2]).

E. coli

The *E. coli* wild-type strain K12 DSM 498 was purchased from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). Bacteria from glycerol stocks were incubated on minimal medium agar plates for at least 1 day. Minimal medium was adapted from *C. glutamicum* to *E. coli* [2]. Cells were transferred to 20 ml of minimal medium and cultivated approximately 16 h at 37 °C and 220 rpm. Two to three flasks, each containing 100 ml of minimal medium, were prepared and inoculated with the overnight culture to an OD_{600} of 0.03–0.05. The cells were grown until an OD_{600} of 1.8 was achieved. Before sampling, the flasks were combined. For metabolome analysis, 16 ml was taken for each sample.

S. cerevisiae

The *S. cerevisiae* CEN.PK 113-7D strain (kindly provided by P. Kötter, University of Frankfurt, Germany) was streaked out from glycerol stocks on YPD agar plates and incubated at 30 °C for 3 days. Several colonies were inoculated in 20 ml of YPD medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose) in baffled shaking flasks and incubated at 30 °C and 200 rpm for 9 h before being diluted 1:10 in 20 ml of fresh minimal medium with 12.5 g/L glucose [27]. This overnight culture was incubated for 14–16 h. Subsequently, cells were resuspended in 50 ml of fresh minimal medium to an OD_{600} of 1 and incubated for 5 h until an OD_{600} of 2.5 was achieved. Four flasks of main culture were incubated and were combined before harvesting. For metabolome analysis, 10 ml was taken for each sample.

Quenching methods

The samples harvested in parallel were further treated according to the different protocols described in the following sections. Standardized conditions for all quenching procedures were used. To verify a complete quenching of the metabolism, separately harvested samples were kept in quenching solution at the defined temperature for 30 min and then further treated.

For comparison purposes, cell dry weight was kept equal between experiments of an organism. Furthermore, cell dry weight among the three different organisms was also adjusted to equal values. This resulted in differences in harvested sample volumes for the strains. Volumes (sample and quenching solution) for *E. coli* and *S. cerevisiae* were reduced to half due to limited tube capacity.

All supernatants were stored at −20 °C for the analysis of the exometabolome to detect possible cell leakage.

Unquenched method

The unquenched samples were prepared according to our published standard protocol [2,26]. Cultures were harvested by centrifugation at 3904*g* and 4 °C and were washed two times with cold (4 °C) 0.9% (w/v) sodium chloride solution. The centrifugation was repeated after each washing step.

Similar to the quenched probes, separate samples were held on ice for 30 min prior to centrifugation and washing and were then treated following the above-mentioned protocol.

Cold ethanol quenching

For each organism, part of the cell suspension was transferred to a 50-ml Falcon tube containing the same volume of precooled (−20 °C) 40% (v/v) ethanol–sodium chloride solution (0.8%, w/v) and mixed instantly by inversion. Subsequently samples were cooled down to approximately −5 to −8 °C in a −30 °C isopropanol bath and centrifuged at 3940*g* and −16 °C for 5–7 min.

To evaluate the influence of washing, the pellet was subjected to zero to two washing steps followed by resuspension in fresh quenching solution using the same volume as before.

To evaluate the quenching efficiency, quenched cells were held at −5 to −8 °C for 30 min before first centrifugation following the above-mentioned protocol.

Cold methanol quenching

For each organism, part of the cell suspension was transferred to a 50-ml Falcon tube containing the double volume of precooled (−50 °C) 60% methanol solution and mixed quickly by inversion [7,9]. Subsequently, samples were centrifuged at 10,000*g* and −9 °C for 5–7 min.

Cold glycerol–saline quenching

For each organism, part of the cell suspension was transferred to a 50-ml Falcon tube containing the fourfold volume of precooled (−20 °C) glycerol–sodium chloride solution (3:2, 13.5 g/L). The suspension was mixed vigorously for 2 s, kept in the −30 °C isopropanol bath for 5 min, and finally centrifuged at 18,000*g* and −9 °C for 10–20 min. In the case of *E. coli,* the sample needed to be divided into two equal parts and combined again during the washing step because of the total volume that exceeded the volume of the Falcon tube.

The pellets were resuspended in 2 ml of precooled (−20 °C) washing solution (glycerol–sodium chloride solution, 1:1, 13.5 g/L) and again centrifuged at 18,000*g* and −9 °C for 10–20 min [25].

To reduce the high glycerol concentration, an additional washing step was added in a separate experiment for further improvement. The pellet was resuspended in 5 ml of precooled (4 °C) 0.9% (w/v) sodium chloride solution, inverted, and subsequently centrifuged at 18,000*g* for 3 min.

Metabolite extraction and derivatization

For all experiments, we used our established protocol for the extraction of intracellular metabolites followed by a two-step derivatization to estimate changes that result from the quenching procedure rather than from the extraction procedure [2,26]. With this method, we are able to analyze a large number of metabolites from widely differing substance classes such as sugars, phosphorylated intermediates, organic acids, amino acids, and nucleosides [2].

Subsequently, cells of the different organisms were resuspended, corresponding to the harvested cell amount, in 1.5 ml of ethanol containing 60 μl of a 0.2-mg/ml ribitol solution (*C. glutamicum*) or in 750 μl of ethanol containing 30 μl of a 0.2-mg/ml ribitol solution (*E. coli* and *S. cerevisiae*), and cells were incubated in an ultrasonic bath for 15 min at 70 °C for cell lysis and metabolite extraction. Samples were cooled for approximately 2 min on ice. Then 1.5 ml (*C. glutamicum*) or 750 μl (*E. coli* and *S. cerevisiae*) of water was added and mixed, followed by the addition of 1 ml of chloroform. The samples were shaken vigorously for chloroform extraction of the apolar phase. After phase separation by centrifugation (3904*g,* 5 min), 800 μl of the polar phase was taken, transferred to a conically shaped glass vial, and then dried in a vacuum concentrator overnight [2,26].

The two-step derivatization procedure (methoxymation using a methoxyamine hydrochloride solution with a concentration of 20 mg/ml in pyridine followed by silylation applying MSTFA) was done automatically using a MPS2 Twister autosampler (Gerstel, Mühlheim an der Ruhr, Germany) equipped with a 10-μl syringe, ensuring no differences in endurance until measurement as described before [26].

Gas chromatography–mass spectrometry analysis

Samples were analyzed using an AccuTOF mass spectrometer (JEOL, Eching, Germany) coupled to an Agilent 6890N fast gas chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a DB-5MS column (Agilent J&W Scientific, Folsom, CA, USA), a programmed temperature vaporizer (PTV) injector (Gerstel), and an MPS2 Twister autosampler (Gerstel). Here 2 μl of the derivatized samples was injected (split ratio 1:25). Separation and mass spectrometry took 18 min. All parameters for sample injection, gas chromatography, and mass spectrometry, as well as metabolite identification, were described elsewhere [26].

Results

In this study, we tested the overall performance of three different quenching procedures and compared them with an unquenched standard procedure. Sampling and extraction procedures were the same for all experiments. Consequently, any changes in metabolite levels between unquenched and quenched samples are due to the specific quenching method applied.

Impact of the ionic strength of the quenching solution

Controversial results of the influence of buffer or salt concentration of the quenching solutions on metabolite concentrations can be found in the literature [4,28]. Therefore, we tested different salt concentrations of our own to determine the best one with respect to the highest relative metabolite concentrations achieved.

As a model organism for this step, the gram-negative prokaryote *E. coli* was used. Because of its morphology, it should show the strongest response to changes in the ionic strength given that gram-negative cells are prone to cell damage caused by changes in osmolarity. The quenching solutions contained 40% ethanol and different concentrations of sodium chloride (0.5, 0.8, and 1.5%, w/v). The best results with the highest relative concentrations and a low standard error, as well as good reproducibility, were found for the quenching solution with 0.8% (w/v) sodium chloride. Of 13 identified amino acids, 8 had the highest relative concentration when applying this salt concentration (data not shown). Altogether, approximately 60% of identified metabolites were found in the highest concentrations in samples prepared with 0.8% sodium chloride in the quenching solution. The overall peak area of all identified substances had been highest after applying the quenching solution with 0.5 or 0.8% (w/v) sodium chloride and much lower with 1.5% (w/v) salt concentration. The average standard deviation had been highest with approximately 24% when using the lowest salt concentration. Nevertheless, additional experiments with *C. glutamicum* and *S. cerevisiae* using the decreased sodium chloride concentration (0.5%, w/v) were done but had no effect on their metabolic profiles.

Comparison of the different applied quenching methods

The cold glycerol–saline quenching did not meet our requirements for an applicable quenching method. The protocol took five times longer than the other quenching procedures. Therefore, we tried to reduce the centrifugation time, but due to the high glycerol concentration, this was not always successful. It turned out to be impossible to get rid of the glycerol sticking to the compact pellets at this low temperature. Consequently, we tried to add one more washing step with ice-cold 0.9% (w/v) sodium chloride solution. This led to a reduction of the glycerol but not to complete removal. The obtained chromatograms showed a dominating glycerol peak masking other metabolites (data not shown).

Results for E. coli

With the special morphology of a gram-negative bacterium, *E. coli* was estimated to be the most sensitive organism in our experiments. Consequently, we expected to see the strongest effects of leakage when applying quenching.

Ethanol quenching at −20 °C outperfomed the other two tested procedures, with methanol quenching giving the lowest peak areas. This was especially obvious for the amino acids and several phosphorylated compounds (Table 1). Compared with the ethanol quenching, more than 80% of the identified metabolites showed lower relative concentrations in the methanol quenching experiment and 60% did so in the unquenched experiment.

In the supernatants of the unquenched and methanol-quenched samples, only pyruvate was found in an increased relative concentration (factor of 1.5), corresponding to a two times higher intracellular concentration in the ethanol quenching experiment. Succinate was found in a significantly higher concentration (nearly a factor of 150) in supernatants of the unquenched samples compared with those of samples quenched with both methods. However, in corresponding extracts, it was decreased by factors of 6 (cold ethanol quenching) and 8 (cold methanol quenching) when comparing the quenched samples with the unquenched ones.

In samples quenched with ethanol solution, a higher number of phosphorylated compounds was identified, and these showed higher relative concentrations (Table 1).

On the other hand, it becomes obvious that for a number of amino acids, much lower concentrations in the quenched samples compared with the unquenched method are measured, but this does not correspond to elevated peak areas in the medium. Only two amino acids could be found in the supernatants of the ethanol-quenched samples. Compared with unquenched samples, concentrations of alanine and aspartate in the supernatants after ethanol quenching were reduced by factors of 1.2 and 6.0, respectively. But under these conditions, they were found in increased concentrations in the cell extracts.

Similar observations were made for intermediates of glycolysis, pentose phosphate pathway, and tricarboxylic acid cycle (Table 1).

Lactic acid showed more than a 10-fold increase in the unquenched samples, as well as a 3.5-fold higher relative concentration in the supernatant of these samples, compared with the ethanol method.

Additional experiments were performed adding one or two washing steps to the ethanol quenching method to test whether the large amount of glucose found in the extracts might result from residual medium sticking to the cells after centrifugation. It became immediately obvious that the washing had a strong negative effect on the analysis. Whereas approximately 63% of the identified metabolites showed a higher concentration in the ethanol-quenched samples compared with the unquenched samples after (only) one washing step, 57% of the relative metabolite concentrations were decreased in the quenched samples. Altogether, 72% of the compounds were reduced in concentration after washing. This could result from the shear stress during resuspension and centrifugation, resulting in cell leakage. Not only sugars but also a lot of sugar phosphates and intermediates of the tricarboxylic acid cycle were diminished. A second additional washing step confirmed that trend. Each washing step caused a reduction of metabolite levels affecting more than 70% of the compounds. Therefore, we avoided the extra washing of the cells.

To verify the quenching efficiency of the ethanol quenching method, additional experiments were performed to make sure that the detected differences between ethanol-quenched and unquenched samples were due to (nearly) completely stopped metabolism when applying ethanol quenching. Hardly any changes of metabolite pools were detected after 30 min when applying the ethanol quenching protocol, but metabolite levels changed significantly in the unquenched samples. This result was well reproducible. Approximately 120 metabolites were identified using our metabolite library, leading to a correlation of the logarithmic values of the relative concentrations (calculated according to Pearson) of 0.83 ± 0.02 for the comparison of the unquenched

Table 1
Comparison of relative concentrations of identified amino acids, phosphorylated compounds, and other metabolites in *E. coli* applying the different procedures.

Metabolite	Ratio of ethanol quenching to unquenched method	Ratio of methanol quenching to unquenched method	Ratio of methanol quenching to ethanol quenching
Amino acids			
Alanine	3.336 ± 0733	0.197 ± 0.055	0.063 ± 0.012
Aspartic acid	35.437 ± 21.262	Not detected in MQ	Not detected in MQ
Glutamic acid	0.075 ± 0.010	0.001 ± 0.0001	0.114 ± 0.036
Glycine	0.547 ± 0.043	0.048 ± 0.026	0.088 ± 0.043
Phenylalanine	5.556 ± 0.777	0.141 ± 0.035	0.024 ± 0.006
Threonine	0.105 ± 0.034	Not detected in MQ	Not detected in MQ
Tyrosine	0.014 ± 0.001	0.008 ± 0.001	0.595 ± 0.101
Valine	0.536 ± 0.353	0.014 ± 0.002	0.035 ± 0.008
Phosphorylated compounds			
3-Phosphoglyceric acid	1.238 ± 0.210	Not detected in MQ	Not detected in MQ
6-Phosphogluconate	1.317 ± 0.131	0.009 ± 0.004	0.007 ± 0.003
Adenosine-5′-monophosphate	Not detected in UQ	Not detected in UQ	0.055 ± 0.020
Erythrose-4-phosphate	1.114 ± 0.089	0.022 ± 0.001	0.017 ± 0.0005
Fructose-1,6-bisphosphate	Not detected in UQ	not detected in UQ	0.001 ± 0.0003
Glucose-6-phosphate	1.930 ± 0.115	0.462 ± 0.133	0.231 ± 0.064
Glycerol-3-phosphate	0.989 ± 0.098	0.216 ± 0.047	0.218 ± 0.043
Ribulose-5-phosphate	3.415 ± 0.853	0.109 ± 0.031	0.063 ± 0.018
Uridine-5′-monophosphate	Not detected in UQ	Not detected in UQ	0.025 ± 0.003
Xylulose-5-phosphate	1.316 ± 0.131	0.202 ± 0.044	0.154 ± 0.030
Others			
α-Ketoglutaric acid	0.105 ± 0.019	Not detected in MQ	Not detected in MQ
Citric acid	0.601 ± 0.072	0.053 ± 0.003	0.097 ± 0.019
Fumaric acid	2.369 ± 0.260	0.170 ± 0.045	0.073 ± 0.018
Lactic acid	0.086 ± 0.017	0.079 ± 0.016	0.916 ± 0.229
Malic acid	2.762 ± 0.165	0.015 ± 0.002	0.005 ± 0.001
Pyruvic acid	2.156 ± 0.237	0.678 ± 0.081	0.338 ± 0.033
Succinic acid	0.161 ± 0.011	0.017 ± 0.008	0.121 ± 0.061

Note. In total, 118 metabolites were identified after ethanol quenching (EQ), revealing an overall standard error of 15%. In total, 87 metabolites were found after methanol quenching (MQ) and 108 metabolites were found in unquenched samples, with overall standard errors of 32% (methanol quenching) and 20% (unquenched method).

method (0 and 30 min) and 0.94 ± 0.04 for the ethanol-quenched samples (0 and 30 min). The strongest relative differences in the quenched samples were found for some amino acids (e.g., valine, factor of 8.6; β-alanine, factor of 4.4; glutamate, factor of 3.8; threonine, factor of 2.8) and ribose-5-phosphate (factor of 6.1) being reduced as well as for leucine (factor of 2.8), 2-hydroxybutanoic acid (factor of 2.2), fructose (factor of 1.6), and fructose-1,6-bisphosphate (factor of 1.5) being increased. For the unquenched samples, many more and much higher differences were found, including 2-phosphoglycerate (factor of 50), phosphoenolpyruvate (factor of 45), methionine (factor of 27), glycerate (factor of 15), 3-phosphoglycerate (factor of 15), and uridine-5′-monophosphate (factor of 12) being increased as well as glucose (factor of 14), lactate (factor of 4.6), 6-phosphogluconate (factor of 2.5), glucose-6-phosphate (factor of 2.3), α-ketoglutarate (factor of 2.0), and pyruvate (factor of 1.8) being reduced.

Another important result is the low relative standard error of 15% for the ethanol-quenched samples corresponding to better reproducibility compared with the methanol method with a relative standard error of 32% and the unquenched method with a relative standard error of 20%. The number of identified metabolites was the highest in samples quenched with ethanol and was the lowest in samples quenched with methanol (Fig. 1). In ethanol-quenched samples, approximately 36% more metabolites were found compared with methanol-quenched samples, and more than 9% more metabolites were found when compared with unquenched samples. Altogether, 124 different metabolites were found in the samples when applying our internal library.

As mentioned, the amino acids tyrosine, threonine, glutamate, and valine, as well as the organic acids lactic acid, α-ketoglutaric acid, and succinate, revealed reduced concentrations in samples quenched with ethanol compared with unquenched samples. These strongly decreased (in some cases) values might be due primarily to the ongoing metabolism in the unquenched samples. All of these substances, excluding lactic acid and α-ketoglutaric acid, showed further increases in concentrations in the unquenched samples after being held on ice for 30 min.

Results for C. glutamicum

According to published results from the literature, the metabolism of this organism cannot be quenched without leakage when using a cold alcohol solution [11]. Nevertheless, the cold ethanol and cold methanol quenching solutions were tested and compared with the results of the published unquenched method for this gram-positive bacterium [2].

In agreement with the literature data, methanol quenching showed a loss in relative concentrations for more than 50% of the identified substances compared with the unquenched procedure, although not for all metabolites could an increased amount be found in the supernatant. Due to the high carbon source concentration in the medium and the dilution during quenching, some concentrations might have been below the detection limit. Some metabolites of the tricarboxylic acid cycle (e.g., fumaric acid, malic acid, succinic acid) were found in the supernatant. Also, lactic acid and pyruvic acid, as well as alanine and glycine, were detected. However, no correlation between the compounds in the supernatant and a possible decrease of their levels in the cell extract could be found.

Glycine was found in increased levels in cell extracts of unquenched samples, and a four times higher glycine concentration was found in the supernatant of the unquenched samples compared with ethanol-quenched samples.

A comparison of the different quenching methods showed a better result for the ethanol-quenched samples than for the methanol-quenched samples. Table 2 gives a representative overview of different metabolites (e.g., amino acids, phosphorylated compounds, intermediates of the central pathways). More than 50% of all identified metabolites were found in higher relative concentrations in the ethanol-quenched samples compared with the unquenched ones. In addition, the direct comparison of the two applied quenching methods showed that more than 70% of the compounds had a larger amount in the ethanol method. The strongest losses in the unquenched samples were observed for α-ketoglutarate, succinate, 2-hydroxyglutarate, pyruvate, citrate, lactate, gluconolactone, and glucose (reduction by factors of 2.5–80.0).

A comparison of quenched samples measured instantly with those measured after 30 min showed good correlations of the relative concentrations. The ethanol quenching procedure (0 and 30 min) had a correlation of 0.94 ± 0.01, and the unquenched method (0 and 30 min) had a correlation of 0.94 ± 0.03. This seems to indicate that for this organism most of the enzyme is nearly inactive even in the unquenched samples at 4 °C compared with 30 °C during cell growth. The strongest relative differences in the quenched samples were found for 2-phosphoglycerate (factor of

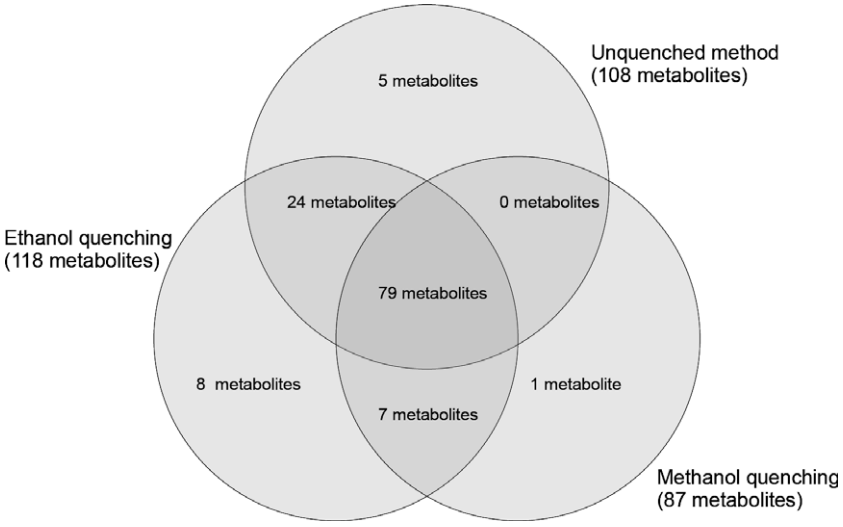

Fig. 1. Venn diagram illustrating the distribution of the 124 identified metabolites resulting from the three methods applied for *E. coli*.

Table 2
Comparison of relative concentrations of identified amino acids, phosphorylated compounds, and other metabolites in *C. glutamicum* applying the different procedures.

Metabolite	Ratio of ethanol quenching to unquenched method	Ratio of methanol quenching to unquenched method	Ratio of methanol quenching to ethanol quenching
Amino acids			
Alanine	1.264 ± 0.189	0.659 ± 0.032	0.536 ± 0.107
Aspartic acid	0.469 ± 0.037	0.183 ± 0.047	0.376 ± 0.101
Glutamic acid	0.728 ± 0.043	0.260 ± 0.020	0.344 ± 0.048
Glycine	0.630 ± 0.207	0.101 ± 0.020	0.159 ± 0.052
Phenylalanine	2.072 ± 0.124	0.736 ± 0.242	0.375 ± 0.097
Tyrosine	1.269 ± 0.190	0.613 ± 0.091	0.443 ± 0.101
Phosphorylated compounds			
2-Phosphoglyceric acid	0.965 ± 0.048	0.334 ± 0.023	0.344 ± 0.024
3-Phosphoglyceric acid	1.152 ± 0.046	0.454 ± 0.049	0.394 ± 0.043
Adenosine-5′-monophosphate	1.595 ± 0.111	0.834 ± 0.075	0.517 ± 0.046
Fructose-1,6-bisphosphate	0.583 ± 0.052	0.224 ± 0.044	0.416 ± 0.133
Glucosamine-6-phosphate	0.943 ± 0.198	0.642 ± 0.112	0.738 ± 0.169
Glucose-6-phosphate	1.233 ± 0.123	1.453 ± 0.188	1.152 ± 0.103
Glycerol-3-phosphate	0.282 ± 0.014	0.093 ± 0.022	1.320 ± 0.330
Mannose-6-phosphate	1.470 ± 0.176	2.708 ± 0.324	1.897 ± 0.360
Phosphoenolpyruvic acid	0.934 ± 0.093	0.685 ± 0.075	0.733 ± 0.095
Uridine-5′-monophosphate	1.514 ± 0.045	0.984 ± 0.059	0.638 ± 0.038
Others			
α-Ketoglutaric acid	2.607 ± 0.286	3.335 ± 0.266	1.251 ± 0.175
Citric acid	21.904 ± 1.752	2.806 ± 0.757	0.129 ± 0.034
Fumaric acid	1.092 ± 0.152	1.743 ± 0.435	1.553 ± 0.434
Lactic acid	29.550 ± 7.387	22.900 ± 5.725	0.775 ± 0.108
Malic acid	1.554 ± 0.170	1.587 ± 0.095	1.030 ± 0.123
N-Acetyl-l-glutamic acid	1.455 ± 0.029	0.909 ± 0.054	0.631 ± 0.031
Pyruvic acid	5.988 ± 0.479	9.646 ± 0.964	1.521 ± 0.167
Succinic acid	2.744 ± 0.356	2.159 ± 0.410	0.787 ± 0.149
Trehalose	0.916 ± 0.027	0.658 ± 0.019	0.723 ± 0.014

Note. In total, 151 metabolites were identified after ethanol quenching, revealing an overall standard error of 18%. In total, 146 metabolites were found after methanol quenching and 144 metabolites were found in unquenched samples, with overall standard errors of 23% (methanol quenching) and 14% (unquenched method).

1.7), 3-phosphoglycerate (factor of 1.5), tryptophan (factor of 1.9), and glycine (factor of 1.7) being increased as well as for glutamine (factor of 6.6), aspartate (factor of 2.2), and lactose (factor of 1.5) being reduced. For the unquenched samples, the strongest relative differences were found for *O*-acetyl-serine (factor of 5.4), malonate (factor of 2.4), pyruvate (factor of 2.4), glycerol-3-phosphate (factor of 1.9), fumarate (factor of 1.8), and malate (factor of 1.6) being increased as well as for adenine (factor of 2.7) and fructose-1,6-bisphosphate (factor of 2.0) being reduced. The variance with a value of ± 0.03 is slightly higher for the unquenched method.

The average relative standard error of the ethanol quenching method was 18%, whereas the methanol quenching showed an error of 23%. The unquenched method for this organism led to a relative error of 14%.

Altogether, 160 different metabolites were found in applying our internal library. Fig. 2 gives an overview of the distribution of the compounds. The difference in the number of identified compounds is much smaller for *C. glutamicum* than for *E. coli*. More than 3% more metabolites were identified in samples quenched with ethanol compared with those quenched with methanol, and approximately 5% more metabolites were identified

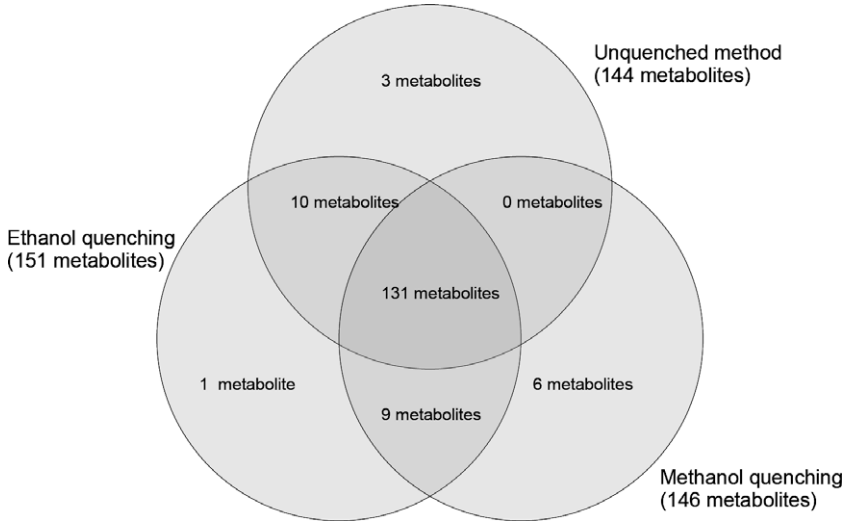

Fig. 2. Venn diagram illustrating the distribution of the 160 identified metabolites resulting from the three methods applied for *C. glutamicum*.

in samples quenched with ethanol compared with unquenched samples.

Results for S. cerevisiae

S. cerevisiae was chosen for comparison for several reasons: (i) it is a well-established model organism for eukaryotes, (ii) information on the reaction of the cells to quenching procedures are evaluable, and (iii) the quenching procedure using cold methanol solution was first established for yeast [9].

Some representative results of the described experiments comparing the two quenching methods and the unquenched method are shown in Table 3. Both quenching methods produced good results. Approximately 60% of all identified metabolites in the quenched samples showed increased concentrations compared with the unquenched procedure. The direct comparison of both quenching methods gave an increased level for 60% of the metabolites in samples quenched with ethanol.

Again the supernatant was analyzed to detect possible cell leakage during quenching. The same results as already described for the other organisms were achieved. Only metabolites of the central metabolism (e.g., pyruvic acid, lactic acid, oxalic acid) were found, but these compounds also showed increased concentrations in the corresponding cell extracts.

In total, 152 metabolites were identified using our internal metabolite library. For the tested procedures, an average standard error for the mean values of all detected relative concentrations of 16% was obtained for the unquenched method, whereas corresponding values of 10 and 17% were obtained for the ethanol quenching and methanol quenching procedures, respectively. The distribution of the detected compounds is shown in Fig. 3. The Venn diagram illustrates the previously described results, showing nicely that there are only small differences among the tested methods.

Again the efficiency of quenching was confirmed by applying the incubation experiment described for *E. coli* and *C. glutamicum*. Only minor differences were detected between the directly prepared samples and the samples processed 30 min later. The correlation coefficients of the logarithmic values for the approximately 150 metabolites found with our internal library were 0.95 ± 0.01 for the unquenched samples (0 and 30 min) and 0.96 ± 0.01 for the quenched samples (0 and 30 min). Again only a slightly better result for the quenched samples was achieved. The reason is probably the same as already described for *C. glutamicum;* it seems that most enzymes' functions in *S. cerevisiae* can be stopped when keeping the samples on ice. Consequently, only few differences could be noticed even when comparing the unquenched samples after 0 min with those after 30 min. Changes in the metabolism might appear only during the very first moments before the temperature dropped to 0 °C. In fact, it is clear that changes occur because there are obvious differences between the quenched and unquenched samples. The strongest relative differences in the quenched samples were found for hexadecanoic acid (factor of 2.0), lactose (factor of 1.9), pyruvate and glycerol (both a factor of 1.7), and carbonate (factor of 1.5) being increased as well as for 3-phosphoglycerate, 6-phosphogluconate, pyrophosphate, and leucine (factors of ~5.0) being reduced. In the unquenched samples, the strongest relative differences were found for pyrophosphoric acid (factor of 3.1), glucosamine-6-phosphate (factor of 2.9), inosine (factor of 2.1), carbonate (factor of 1.8), malonate (factor of 1.5), and fumarate (factor of 1.4) being increased as well as for erythrose-4-phosphate (factor of 12.0), nicotinic acid (factor of 6.7), and 6-phosphogluconate (factor of 6.2) being reduced.

Table 3
Comparison of relative concentrations of identified amino acids, phosphorylated compounds, and other metabolites in *S. cerevisiae* applying the different procedures.

Metabolite	Ratio of ethanol quenching to unquenched method	Ratio of methanol quenching to unquenched method	Ratio of methanol quenching to ethanol quenching
Amino acids			
Alanine	1.035 ± 0.041	0.916 ± 0.054	0.889 ± 0.044
Aspartic acid	1.455 ± 0.218	1.147 ± 0.114	0.817 ± 0.024
Glutamic acid	1.148 ± 0.068	1.089 ± 0.065	0.930 ± 0.027
Glutamine	1.238 ± 0.185	0.846 ± 0.169	0.683 ± 0.109
Leucine	0.715 ± 0.042	0.612 ± 0.048	0.832 ± 0.116
Lysine	1.132 ± 0.101	1.060 ± 0.106	0.925 ± 0.046
Phenylalanine	0.878 ± 0.061	0.940 ± 0.075	1.070 ± 0.053
Proline	0.767 ± 0.023	0.661 ± 0.039	0.874 ± 0.061
Serine	1.517 ± 0.106	1.483 ± 0.281	0.960 ± 0.105
Threonine	1.083 ± 0.021	1.016 ± 0.050	0.936 ± 0.037
Tryptophane	1.585 ± 0.396	1.511 ± 0.392	0.937 ± 0.037
Tyrosine	0.999 ± 0.049	0.952 ± 0.047	0.950 ± 0.038
Valine	0.874 ± 0.017	0.825 ± 0.041	0.946 ± 0.047
Phosphorylated compounds			
3-Phosphoglyceric acid	0.078 ± 0.0007	0.111 ± 0.002	1.414 ± 0.438
Erythrose-4-phosphate	0.905 ± 0.045	0.956 ± 0.066	1.068 ± 0.106
Glycerol-3-phosphate	0.922 ± 0.027	1.116 ± 0.167	1.261 ± 0.239
Ribulose-5-phosphate	1.010 ± 0.050	1.508 ± 0.241	1.562 ± 0.281
Xylulose-5-phosphate	0.984 ± 0.049	1.380 ± 0.220	1.445 ± 0.289
Others			
Citric acid	1.399 ± 0.111	1.322 ± 0.198	0.922 ± 0.073
Fumaric acid	0.758 ± 0.022	1.023 ± 0.071	1.351 ± 0.108
Lactic acid	1.026 ± 0.041	0.862 ± 0.051	0.847 ± 0.042
Malic acid	0.847 ± 0.008	0.626 ± 0.018	0.740 ± 0.029
Pyruvic acid	2.047 ± 0.655	0.695 ± 0.201	0.458 ± 0.050
Succinic acid	0.619 ± 0.018	0.585 ± 0.023	0.966 ± 0.057

Note. In total, 150 metabolites were identified after ethanol quenching, revealing an overall standard error of 10%. In total, 151 metabolites were found after methanol quenching and 148 metabolites were found in unquenched samples, with overall standard errors of 17% (methanol quenching) and 16% (unquenched method).

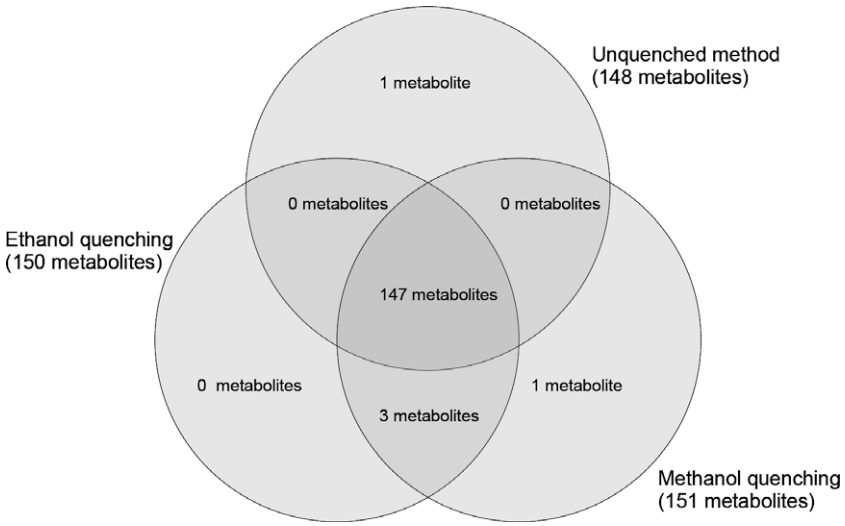

Fig. 3. Venn diagram illustrating the distribution of the 152 identified metabolites resulting from the three methods applied for *S. cerevisiae*.

Discussion

The applicability and influence of three different quenching methods—ethanol (this article), methanol [9], and glycerol–saline quenching [25]—and of the unquenched standard method [2] on the results of a metabolome analysis were compared. The procedures were tested for three different organisms: *E. coli, C. glutamicum,* and *S. cerevisiae.* Metabolite extraction and derivatization, as well as gas chromatography–mass spectrometry analysis, were performed according to our established protocol [26] to avoid any differences resulting from different protocols.

Due to the chemical diversity of metabolites, several extraction protocols exist [5]. The extraction with ethanol in an ultrasonic bath at 70 °C is our standard procedure, allowing the parallel quantification of up to 180 different compounds for *C. glutamicum* when applying our internal library, which now comprises more than 500 derivatives [26]. For the purpose of establishing a new quenching method, this procedure was used as a basis even given the known fact of limited metabolite recovery of any single extraction method.

Although in one study better results for washing of *E. coli* cells were obtained with a salt concentration of 2.6% [28], in the experiments described here we observed optimal detection for metabolites of the central metabolic pathways and amino acids when applying a quenching solution with the physiological salt concentration of 0.8% (w/v). Most experiments in this respect were performed with *E. coli* because its morphology is likely to show the strongest response to osmotic stress. It should be noted here that experiments with a decreased sodium chloride concentration (0.5%, w/v) had an adverse effect only on *E. coli* and not on the other tested organisms.

Additional washing steps showed a strong decrease in metabolite concentrations for *E. coli.* This could be a result from cell leakage occurring during the resuspension and centrifugation of the cells, causing shear stress. Furthermore, the overall estimated error increased and reproducibility decreased.

Less than satisfying results were achieved in experiments using the cold glycerol–saline method. Due to the increased viscosity of the solution, the protocol steps were too time-consuming and a large amount of glycerol was sticking to the cells. Another problem with the glycerol is its hygroscopicity, leading to problems during silylation with our method [2,26]. Silylation with MSTFA [29] requires water-free samples. But the use of methyl chloroformate (MCF), as described in Ref. [25], would decrease the number of different detectable metabolites because it is suitable for derivatizing only a limited number of different chemical compound classes (e.g., amino acids, organic acids).

For the evaluation of the ethanol quenching method proposed in this article, the distribution of the overall number of identified substances was determined and the supernatant was analyzed to verify whether cell leakage had occurred. Also, additional experiments were performed to verify the (nearly) complete quenching of the metabolism. As expected, successful quenching of the metabolism could be confirmed. Although *E. coli, C. glutamicum,* and *S. cerevisiae* are very different in their morphology, for all three organisms the best results were obtained when applying the ethanol quenching procedure.

As expected, *E. coli* as the gram-negative organism showed the strongest response; whereas approximately 70% of all estimated relative metabolite concentrations were reduced in samples of the methanol quenching method compared with the unquenched method, approximately 60% of the metabolite levels were found in higher concentrations when ethanol quenching was applied. The direct comparison of the methanol and ethanol quenching methods showed increased amounts for more than 80% of all quantified compounds and a 36% increase in the number of detectable metabolites. This was an unexpectedly strong improvement of the results resulting from a quenching procedure. Recently, the methanol quenching method was described as a suitable method for *E. coli* despite cell leakage [12]. Our findings do not support this; instead, they suggest the use of the ethanol quenching procedure for the metabolome analysis of *E. coli.* In addition to the better metabolite recovery, ethanol quenching resulted in a significantly lower (less than half the value) overall standard error of 15% for a larger number of compounds (compared with 32% for methanol quenching), showing better reproducibility and stability of the method. In particular, the number of determined sugar phosphates and amino acids was the highest in samples prepared according to the ethanol quenching protocol.

Similar results were obtained for *C. glutamicum.* Approximately 51% of all quantified metabolites were detected in increased levels when compared with unquenched samples, and 70% were enhanced when compared with results from samples quenched with methanol. Again the recovery rates, as well as the number of identified compounds, were larger when ethanol quenching was applied. Furthermore, the good reproducibility and stability of this

method used for quenching of *C. glutamicum* were confirmed by the overall standard error of all detected metabolites of 18%, whereas methanol quenching showed a corresponding value of 23%. The observation of reduced amino acid levels and concentrations of phosphorylated compounds in samples of *C. glutamicum* quenched with cold methanol described in the literature [11] could be confirmed only partly (Table 2). Amino acid concentrations were decreased, but results for phosphorylated substances varied when methanol-quenched samples were compared with unquenched ones. Opposite to the methanol results, samples quenched with cold ethanol solution showed increased levels for some amino acids, as well as phosphorylated compounds, in comparison with unquenched samples.

The quenching method using cold methanol had been described previously for *S. cerevisiae* [9]. In our experiments with *S. cerevisiae,* this method led to good results as well. Nevertheless, application of the cold ethanol quenching solution proved to be superior. The direct comparison of both methods showed an increased relative concentration for 60% of the metabolites, and 64% of metabolite levels were enhanced when compared with the unquenched standard method. In addition, the overall standard error of 152 identified compounds was lowest in samples quenched with ethanol at only 10%, confirming very good reproducibility and stability of the method. In contrast, for methanol quenching, an error of 17% was obtained.

The experiments for the detection of cell leakage gave inconsistent results. A direct relation for substances with reduced levels in the extract and corresponding increased levels in the supernatant, which would be a clear indication of cell leakage, could not be detected. Mainly intermediates of the tricarboxylic acid cycle or amino acids, as was already mentioned in the literature [6], were detected in the supernatant. But in many cases, these metabolites were found in the corresponding cell extracts in increased values as well. In these cases, the extracellular metabolites could also originate from secretion or from lysis of cells always present during cultivation. The observed differences between metabolite concentrations of the identified metabolites in the different methods cannot be due primarily to cell leakage.

To further confirm the good results obtained with ethanol quenching, additional experiments were performed to verify the complete stop of metabolism. Therefore, contact time of cells and quenching solution was extended (30 min). For comparison reasons, unquenched samples were held on ice for 30 min as well. For all three organisms, very clear results were obtained. When applying ethanol quenching, the metabolism was nearly completely stopped. The estimated correlation coefficient between the logarithmic values of the mean of all identified relative metabolite concentrations was 0.94 or even higher (0.96 for *S. cerevisiae*) along with a very low variation of 0.01 (for *C. glutamicum* and *S. cerevisiae*) or 0.04 (for *E. coli*). In *E. coli,* some amino acids (e.g., valine, factor of 8.6; glutamate, factor of 3.8; aspartate, factor of 2.5), ribose-5-phosphate (factor of 6.1), and fructose-1,6-bisphosphate (factor of 1.5) showed the strongest relative differences in this comparison. In *C. glutamicum,* 2- and 3-phosphoglycerate and some amino acids (tryptophan, glycine, and aspartate) were found to differ (all below a factor of 2.0). For *S. cerevisiae,* differences for 3-phosphoglycerate and 6-phosphogluconate (both a factor of 5.0), hexadecanoic acid, lactose, pyruvate, and glycerol (all below a factor of 2.0) were identified.

E. coli showed the greatest differences. The metabolic profile of unquenched cells kept on ice for 30 min showed a correlation of just 0.83 to the profile obtained before this period. For the other two organisms, the correlation coefficients of the unquenched samples did not decrease so much, with 0.94 ± 0.03 for *C. glutamicum* and 0.95 ± 0.01 for *S. cerevisiae.* The results suggest that the first part of the quenching procedure, namely the cooling of the cells, is the critical step. Cells must be cooled down in a few seconds. The difference observed for *E. coli* could be a result of the higher cultivation temperature of 37 °C compared with 30 °C for *C. glutamicum* and *S. cerevisiae.*

An overall statistical evaluation of the performance of the three methods applied to the three organisms was done taking into consideration the following criteria: (i) the number of compounds found, (ii) the average statistical error of the measurements, and (iii) the number of compounds found with the highest peak areas. This leads to nine comparisons (three organisms and three criteria) of the three methods. In this comparison, the ethanol quenching method gave the best result six times and the second-best result three times; the methanol quenching method gave the best result one time, the second-best result two times, and the worst result six times; and the unquenched sample gave the best result two times, the second-best result four times, and the worst result three times.

Conclusion

A reproducible, stable, and fast quenching method using a cold ethanol solution led to improved results for three tested organisms—*E. coli, C. glutamicum,* and *S. cerevisiae*—covering a gram-negative prokaryote, a gram-positive prokaryote, and a eukaryote. The results clearly underline that the method is applicable not only for different types of organisms but also for routine use.

Acknowledgment

The authors thank the German research ministry (BMBF) for financial support.

References

[1] A. Buchholz, J. Hurlebaus, C. Wandrey, R. Takors, Metabolomics: quantification of intracellular metabolite dynamics, Biomol. Eng. 19 (2002) 5–15.
[2] S. Strelkov, M. von Elstermann, D. Schomburg, Comprehensive analysis of metabolites in *Corynebacterium glutamicum* by gas chromatography/mass spectrometry, Biol. Chem. 385 (2004) 853–861.
[3] S.G. Villas-Boas, S. Mas, M. Akesson, J. Smedsgaard, J. Nielsen, Mass spectrometry in metabolome analysis, Mass Spectrom. Rev. 24 (2005) 613–646.
[4] A.B. Canelas, C. Ras, A. ten Pierick, J.C. van Dam, J.J. Heijnen, W.M. Van Gulik, Leakage-free rapid quenching technique for yeast metabolomics, Metabolomics 4 (2008) 226–239.
[5] M.R. Mashego, K. Rumbold, M.D. Mey, E. Vandamme, W. Soetaert, J.J. Heijen, Microbial metabolomics: past, present, and future methodologies, Biotechnol. Lett. 29 (2007) 1–16.
[6] M.O. Loret, L. Pedersen, J. Francois, Revised procedures for yeast metabolites extraction: application to a glucose pulse to carbon-limited yeast cultures, which reveals a transient activation of the purine salvage pathway, Yeast 24 (2007) 47–60.
[7] M.A. Hans, E. Heinzle, C. Wittmann, Quantification of intracellular amino acids in batch cultures of *Saccharomyces cerevisiae*, Appl. Microbiol. Biotechnol. 56 (2001) 776–779.
[8] B. Moritz, K. Striegel, A.A. de Graaf, H. Sahm, Kinetic properties of the glucose-6-phosphate and 6-phosphogluconate dehydrogenases from *Corynebacterium glutamicum* and their application for predicting pentose phosphate pathway flux in vivo, Eur. J. Biochem. 267 (2000) 3442–3452.
[9] W. de Koning, K. van Dam, A method for the determination of changes of glycolytic metabolites in yeast on a subsecond time scale using extraction at neutral pH, Anal. Biochem. 204 (1992) 118–123.
[10] B. Gonzalez, J. Francois, M. Renaud, A rapid and reliable method for metabolite extraction in yeast using boiling buffered ethanol, Yeast 13 (1997) 1347–1356.
[11] C. Wittmann, J.O. Krömer, P. Kiefer, T. Binz, E. Heinzle, Impact of the cold shock phenomenon on quantification of intracellular metabolites in bacteria, Anal. Biochem. 327 (2004) 135–139.
[12] C.L. Winder, W.B. Dunn, S. Schuler, D. Broadhurst, R. Jarvis, G.M. Stephens, R. Goodacre, Global metabolic profiling of *Escherichia coli* cultures: an evaluation of methods for quenching and extraction of intracellular metabolites, Anal. Chem. 80 (2008) 2939–2948.
[13] M. Oldiges, S. Lütz, S. Pflug, K. Schroer, N. Stein, C. Wiendahl, Metabolomics: current state and evolving methodologies and tools, Appl. Microbiol. Biotechnol. 76 (2007) 495–511.
[14] N.B.S. Jensen, K.V. Jokumsen, J. Villadsen, Determination of the phosphorylated sugars of the Embden–Meyerhoff–Parnas pathway in *Lactococcus lactis* using

fast sampling technique and solid phase extraction, Biotechnol. Bioeng. 63 (1999) 356–362.
[15] U. Schäfer, W. Boos, R. Takors, D. Weuster-Botz, Automated sampling device for monitoring intracellular metabolite dynamics, Anal. Biochem. 270 (1999) 88–96.
[16] A. Buchholz, R. Takors, C. Wandrey, Quantification of intracellular metabolites in *Escherichia coli* K12 using liquid chromatographic-electrospray ionization tandem mass spectrometric techniques, Anal. Biochem. 295 (2001) 129–137.
[17] J.I. Castrillo, A. Hayes, S. Mohammed, S.J. Gaskell, S.G. Oliver, An optimized protocol for metabolome analysis in yeast using direct infusion electrospray mass spectrometry, Phytochemistry 62 (2003) 929–937.
[18] S.G. Villas-Boas, J. Hojer-Pedersen, M. Akesson, J. Smedsgaard, J. Nielsen, Global metabolite analysis of yeast: evaluation of sample preparation methods, Yeast 22 (2005) 1155–1169.
[19] S. Buziol, I. Bashir, A. Baumeister, W. Claaßen, N. Noisommit-Rizzi, W. Mailinger, M. Reuss, New bioreactor-coupled rapid stopped-flow sampling technique for measurements of metabolite dynamics on a subsecond time scale, Biotechnol. Bioeng. 80 (2002) 632–636.
[20] M.R. Mashego, W.M. van Gulik, J.L. Vinke, J.J. Heijnen, Critical evaluation of sampling techniques for residual glucose determination in carbon-limited chemostat culture of *Saccharomyces cerevisiae*, Biotechnol. Bioeng. 83 (2003) 395–399.
[21] D. Weuster-Botz, Sampling tube device for monitoring intracellular metabolite dynamics, Anal. Biochem. 246 (1997) 225–233.
[22] G. Larsson, M. Törnkvist, Rapid sampling, cell inactivation, and evaluation of low extracellular glucose concentrations during fed-batch cultivation, J. Biotechnol. 49 (1996) 69–82.
[23] A.M. Cook, E. Urban, H.G. Schlegel, Measuring the concentrations of metabolites in bacteria, Anal. Biochem. 72 (1976) 191–201.
[24] F. Letisse, N.D. Lindley, An intracellular metabolite quantification technique applicable to polysaccharide-producing bacteria, Biotechnol. Lett. 22 (2000) 1673–1677.
[25] S.G. Villas-Boas, P. Bruheim, Cold glycerol–saline: the promising quenching solution for accurate intracellular metabolite analysis of microbial cells, Anal. Biochem. 370 (2007) 87–97.
[26] J. Börner, S. Buchinger, D. Schomburg, A high-throughput method for microbial metabolome analysis using gas-chromatography/mass spectrometry, Anal. Biochem. 367 (2007) 143–151.
[27] A.J. Saldanha, M.J. Brauer, D. Botstein, Nutritional homeostasis in batch and steady-state culture of yeast, Mol. Biol. Cell 15 (2004) 4089–4104.
[28] C.J. Bolten, P. Kiefer, F. Letisse, J.-C. Portais, C. Wittmann, Sampling for metabolome analysis of microorganisms, Anal. Chem. 79 (2007) 3843–3849.
[29] U. Roessner, C. Wagner, J. Kopka, R.N. Trethewey, L. Willmitzer, Simultaneous analysis of metabolites in potato tuber by gas chromatography–mass spectrometry, Plant J. 23 (2000) 131–142.