

Appropriate sampling for intracellular amino acid analysis in five phylogenetically different yeasts

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Abstract Methanol quenching and fast filtration, the two most common sampling protocols in microbial metabolome analysis, were validated for intracellular amino acid analysis in phylogenetically different yeast strains comprising *Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, *Pichia pastoris*, *Schizosaccharomyces pombe* and *Zygosaccharomyces bailii*. With only few exceptions for selected amino acids, all yeasts exhibited negligible metabolite leakage during quenching with 60% cold buffered methanol. Slightly higher leakage was observed with increasing methanol content in the quenching solution. Fast filtration resulted in identical levels for intracellular amino acids in all strains tested. The results clearly demonstrate the validity of both approaches for leakage-free sampling of amino acids in yeast.

Keywords Fast filtration · Metabolome · Methanol quenching · Yeast

Introduction

Sample preparation is considered one of the limiting steps in microbial metabolome analysis. Eukaryotes and prokaryotes and even different species within each kingdom behave differently during sampling for the analysis of metabolites so that protocols developed for a specific group of microorganisms cannot be simply adopted. The most popular method is based on rapid sampling into a cold aqueous methanol solution since it allows a reliable separation of intra- and extracellular metabolites via centrifugation while the metabolism is effectively quenched in the sub second time scale. The latter is crucial to effectively stop the metabolism due to high turn over of intracellular metabolites. Alternatively, fast filtration has been applied, also providing such separation, but limited to some extent due to elevated time intervals of a few seconds prior to extraction. For gram-positive and gram-negative bacteria it was shown that methanol quenching causes tremendous unspecific leakage of intracellular metabolites into the methanol solution during the quenching step and is thus not applicable for metabolome sampling (Bolten et al. 2007). Fast filtration, however, does not result in loss of metabolites (Wittmann et al. 2004). For yeasts, our knowledge on appropriate sampling for metabolite analysis is basically limited to *Saccharomyces cerevisiae*. This species has however been studied in great detail (de Koning and van Dam 1992; Lange et al. 2001; Loret et al. 2007; Saez and Lagunas 1976; Villas-Boas and Bruheim 2007;

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Wallace et al. 1994; Weibel et al. 1974). Among these studies, glucose 6-phosphate, ATP and NAD⁺ were detected in the supernatant of methanol-quenched yeast cells, but could be attributed to originate from the culture medium (Gonzalez et al. 1997). Subsequently it was shown that quenching with 60% buffered methanol does not lead to a leakage of intracellular amino acids (Hans et al. 2001). Vilas-Boas and co-workers observed an influence of the composition of the quenching solution on the extent of leakage (Villas-Boas et al. 2005). Fast filtration, originally developed for prokaryotes, has not been tested for yeast, but is regarded as promising sampling technique. Yeast species other than *S. cerevisiae* have not been systematically studied. In the present work we investigate the suitability of methanol quenching and fast filtration for metabolite analysis in the yeast strains *Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, *Pichia pastoris*, *Schizosaccharomyces pombe* and *Zygosaccharomyces bailii*. The study focuses on the analysis of intracellular amino acids. These comprise a group of metabolites with different physico-chemical properties and high significance in biological studies.

Materials and methods

Organisms and growth conditions

Saccharomyces cerevisiae ATCC 32167 and *Kluyveromyces marxianus* ATCC 26548 were used. *Pichia pastoris* GS115, *Schizosaccharomyces pombe* PW260 and *Zygosaccharomyces bailii* DSM 12864 were kindly donated by Manfred Schmitt (Saarland University, Germany). *Saccharomyces cerevisiae* and *K. marxianus* were cultivated in minimal medium (Hans et al. 2001). The medium was additionally supplemented with 5 mg yeast extract l⁻¹ and 10 mg tryptone l⁻¹ for growth of the other strains. All cultivations were carried out in duplicate at 30°C and 230 rpm on a rotary shaker (shaking diameter 50 mm, Multitron II, Infors AG, Bottmingen-Basel, Switzerland).

Sampling of cultivation supernatant

Cell suspension (2 ml) was sucked into a 2 ml plastic syringe and directly squeezed through a sterile filter

(polyvinylidene fluoride, 0.2 µm pore size, Roth, Karlsruhe, Germany).

Sampling of intracellular amino acids

For metabolite sampling with cold methanol quenching 5 ml cell suspension was sucked into a plastic syringe, directly injected into a pre-cooled Falcon tube filled with 10 ml 60% (v/v) buffered methanol (−58°C, 10 mM HEPES), immediately mixed vigorously and centrifuged for 5 min at 10,000×g and −19°C (Biofuge Stratos, Kendro Laboratory Products, Langensfeld, Germany). By this approach, metabolism is stopped within 0.1 s (de Koning and van Dam 1992). Further tests were performed with varied quenching solution. In contrast to a previous study with yeast, comparing different quenching solutions, a short centrifugation time of 5 min was always applied, since this has proven beneficial with respect to minimization of metabolite leakage (Villas-Boas et al. 2005). The temperature of the quenched sample was always below −20°C as verified by measurement with a digital thermometer. For sampling via fast filtration 2 ml cell suspension was harvested by vacuum filtration (cellulose nitrate filter, 0.2 µm, 25 mm, Sartorius, Göttingen, Germany) and washed with 15 ml fresh minimal medium (room temperature). The filtration procedure including the washing step until the metabolite extraction was finished in less than 30 s. In each experiment sampling was carried out fourfold in parallel.

Intracellular metabolite extraction

Cells harvested via methanol quenching or via fast filtration as described above were incubated in 2 ml α-aminobutyrate solution (200 µM) for 15 min at 100°C for metabolite extraction. Subsequently, the extracts were cooled on ice, transferred into 2 ml tubes and centrifuged (5 min, 16,000×g, 4°C, Biofuge fresco, Kendro Laboratory Products, Langensfeld, Germany) to remove cell debris. The obtained extract was directly used for metabolite quantification.

Sample concentration

Due to the inherently low concentration in these fractions, culture broth, methanol quenching

supernatant and filtrate were concentrated by lyophilization prior to analysis. In the case of samples contained solvent, water was added in appropriate amounts to adjust a water content of >75% and keep the samples frozen during the lyophilization process.

Analytics

Cell concentration was assessed by measurement of optical density (OD) and cell dry weight (CDW) as described earlier (Kiefer et al. 2004). The correlation of optical density to CDW was $CDW = 0.53 OD \times g l^{-1}$ for *P. pastoris*, $CDW = 0.62 OD \times g l^{-1}$ for *S. pombe* and $CDW = 0.54 OD \times g l^{-1}$ for *Z. bailii*. The correlation factors for *S. cerevisiae* ($CDW = 0.50 OD \times g l^{-1}$) (Hollemeier et al. 2007) and *K. marxianus* ($CDW = 0.38 OD \times g l^{-1}$) (Wittmann et al. 2002) were previously measured. Amino acids with a primary amino group were quantified by HPLC using α -aminobutyrate as internal standard (Wittmann et al. 2004). The internal standard was added during the metabolite extraction to exclude potential losses during the subsequent steps of e.g. lyophilization, re-suspension or derivatization. Leucine, isoleucine, phenylalanine and valine could be quantified only in cell extracts, but not in supernatant, i.e. the methanol solution (from methanol quenching) and the filtrate (from fast filtration) because of ammonium contained in the medium which interfered with these compounds in the HPLC analysis. Cell concentration was measured via optical density (OD) at 660 nm.

Results

Methanol quenching

Potential metabolite leakage during sampling into the pre-cooled methanol quenching solution and cell separation was assessed by quantifying amino acids in the cell extract and the methanol supernatant. In addition amino acids were quantified in the culture broth to correct the data obtained for the supernatant for molecules not originating from leakage out of the cell interior, but from the broth, and account exactly for the metabolite leakage.

Saccharomyces cerevisiae, as a reference, exhibited negligible loss for all amino acids analyzed (Table 1).

On average, 96% of the amino acids were retained in the cell interior during the quenching. *Kluyveromyces marxianus*, *Z. bailii* and *S. pombe* showed similar results indicating a leakage-free sampling also in these species via methanol quenching. A slightly different behavior was observed for *P. pastoris*. Most amino acids were still almost completely retained within the cell but up to 30% of the two acidic amino acids, aspartate and glutamate, obviously leaked into the quenching supernatant. Alternative quenching solutions were tested for *S. cerevisiae*. Variation of the quenching solution had only a slight effect on the extent of metabolite leakage (Table 2).

The optimal quenching solution was 60% (v/v) buffered methanol, resulting in an average amino acid loss of below 5%. With increasing methanol content in the buffered solution the average leakage gradually increased, but still was below 10% at the highest concentration tested. Un-buffered methanol resulted in slightly higher leakage (7%) as compared to buffered methanol.

Fast filtration

The sampling procedure comprised vacuum filtration of the culture followed by washing of the cells on the filter with sterile medium. Among the different yeast strains tested, *K. marxianus*, *S. pombe* and *P. pastoris* did not reveal significant leakage, but almost exclusively the amino acid pool was recovered from the cell extract. In contrast, substantial loss of certain amino acids during the filtration procedure was observed for *S. cerevisiae* and *Z. bailii*. On average, the relative amino acid loss via leakage into the filtrate was 8% for *S. cerevisiae* and 20% for *Z. bailii*, whereby the most prominent compounds affected were glycine and serine with up to 40% relative loss. This rather surprising leakage for selected yeast strains was further investigated by variation of the washing solution. As exemplified for *S. cerevisiae* utilization of other washing solutions minimized the extent of leakage, whereby interestingly de-ionized water was found optimal (Table 3).

Table 1 Intracellular amino acid concentrations from *S. cerevisiae*, *Z. bailii*, *K. marxianus*, *P. pastoris* and *S. pombe* utilizing fast filtration including washing with fresh medium and methanol quenching with subsequent cell separation. In each experiment sampling was carried out fourfold in parallel. The concentrations are given in $\mu\text{mol (g CDW)}^{-1}$. The limit of detection was $0.1 \mu\text{mol (g CDW)}^{-1}$. Cysteine, methionine and tryptophan were below the detection limit in all samples. Leucine, Isoleucine, Phenylalanine and Valine could not be quantified (ND) in supernatant because of interfering ammonia contained in the medium during the HPLC analysis

Compound	<i>S. cerevisiae</i>						<i>Z. bailii</i>						<i>K. marxianus</i>					
	Fast filtration		MeOH quenching		Fast filtration		MeOH quenching		Fast filtration		MeOH quenching		Fast filtration		MeOH quenching			
	EX	SN	EX	SN	EX	SN	EX	SN	EX	SN	EX	SN	EX	SN	EX	SN		
Alanine	55.4 ± 2.2	4.4 ± 1.2	51.8 ± 2.2	1.4 ± 0.4	37.2 ± 4.1	4.6 ± 3.3	40.6 ± 0.2	1.7 ± 0.4	250.8 ± 5.5	<0.1	250.0 ± 1.3	<0.1	<0.1	<0.1	<0.1	<0.1		
Arginine	90.8 ± 0.4	5.6 ± 1.0	81.6 ± 4.8	1.0 ± 0.2	10.4 ± 0.7	2.0 ± 0.6	10.0 ± 0.2	0.4 ± 0.2	164.2 ± 13.2	<0.1	168.2 ± 10.8	<0.1	<0.1	<0.1	<0.1	<0.1		
Asparagine	8.4 ± 0.2	1.4 ± 0.6	10.0 ± 0.2	0.4 ± 0.2	3.9 ± 0.2	1.3 ± 0.7	4.1 ± 0.2	<0.1	5.5 ± 2.6	<0.1	6.6 ± 0.3	<0.1	<0.1	<0.1	<0.1	<0.1		
Aspartate	35.8 ± 1.6	2.6 ± 0.8	20.4 ± 2.4	1.0 ± 0.2	23.9 ± 2.8	3.1 ± 1.9	29.3 ± 0.7	0.6 ± 0.2	6.1 ± 0.3	<0.1	6.8 ± 0.3	<0.1	<0.1	<0.1	<0.1	<0.1		
Glutamate	175.8 ± 2.6	6.4 ± 2.6	180.6 ± 10.4	3.6 ± 1.4	130.0 ± 2.8	6.7 ± 1.9	132.2 ± 3.1	2.0 ± 0.7	53.9 ± 2.6	<0.1	58.4 ± 2.6	<0.1	<0.1	<0.1	<0.1	<0.1		
Glutamine	129.8 ± 5.0	5.2 ± 2.2	121.0 ± 4.2	2.2 ± 1.0	18.5 ± 0.7	2.8 ± 1.9	20.0 ± 0.6	0.4 ± 0.2	104.7 ± 4.2	<0.1	105.0 ± 2.9	<0.1	<0.1	<0.1	<0.1	<0.1		
Glycine	14.8 ± 0.8	3.6 ± 1.4	9.0 ± 1.6	0.6 ± 0.2	6.5 ± 0.7	5.0 ± 3.5	6.7 ± 0.6	0.6 ± 0.4	7.9 ± 1.3	<0.1	7.9 ± 1.1	<0.1	<0.1	<0.1	<0.1	<0.1		
Histidine	10.6 ± 0.6	0.8 ± 0.2	6.4 ± 0.6	0.2 ± 0.2	2.6 ± 0.2	0.7 ± 0.6	2.8 ± 0.4	<0.1	5.3 ± 0.8	<0.1	6.3 ± 0.5	<0.1	<0.1	<0.1	<0.1	<0.1		
Isoleucine	4.2 ± 0.6	ND	4.2 ± 0.6	ND	0.9 ± 0.2	ND	0.7 ± 0.2	ND	<0.1	ND	<0.1	ND	<0.1	ND	<0.1	ND		
Leucine	2.2 ± 0.4	ND	2.0 ± 1.0	ND	0.9 ± 0.2	ND	0.7 ± 0.4	ND	2.1 ± 0.5	ND	3.4 ± 0.3	ND	2.1 ± 0.5	ND	3.4 ± 0.3	ND		
Lysine	21.2 ± 0.6	<0.1	20.4 ± 1.6	<0.1	8.9 ± 0.9	<0.1	9.3 ± 0.6	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1		
Phenylalanine	0.8 ± 0.2	ND	1.6 ± 0.8	ND	<0.1	ND	<0.1	ND	<0.1	ND	<0.1	ND	<0.1	ND	<0.1	ND		
Serine	9.0 ± 0.6	0.8 ± 0.2	9.0 ± 0.4	0.6 ± 0.4	10.4 ± 0.2	5.4 ± 3.7	11.7 ± 0.9	0.7 ± 0.6	9.7 ± 1.6	<0.1	8.4 ± 0.8	<0.1	<0.1	<0.1	<0.1	<0.1		
Threonine	18.2 ± 0.4	1.8 ± 0.6	17.0 ± 0.6	0.6 ± 0.2	8.3 ± 0.2	1.7 ± 1.1	9.4 ± 0.2	0.2 ± 0.2	6.1 ± 0.3	<0.1	6.6 ± 0.3	<0.1	<0.1	<0.1	<0.1	<0.1		
Tyrosine	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.5 ± 0.3	<0.1	0.8 ± 0.5	<0.1	<0.1	<0.1	<0.1	<0.1		
Valine	5.6 ± 0.6	ND	4.8 ± 1.2	ND	5.0 ± 0.4	ND	5.4 ± 0.4	ND	9.5 ± 4.2	ND	7.4 ± 0.3	ND	9.5 ± 4.2	ND	7.4 ± 0.3	ND		
Compound	<i>P. pastoris</i>						<i>S. pombe</i>											
	Fast filtration		MeOH quenching		Fast filtration		MeOH quenching		Fast filtration		MeOH quenching							
	EX	SN	EX	SN	EX	SN	EX	SN	EX	SN	EX	SN						
Alanine	37.4 ± 2.5	<0.1	40.4 ± 3.2	<0.1	23.9 ± 1.5	<0.1	19.8 ± 1.1	<0.1	50.8 ± 0.5	<0.1	50.8 ± 0.5	<0.1						
Arginine	157.5 ± 4.3	<0.1	162.8 ± 7.4	<0.1	57.4 ± 5.5	<0.1	10.0 ± 0.5	<0.1	10.0 ± 0.5	<0.1	10.0 ± 0.5	<0.1						
Asparagine	18.7 ± 0.8	<0.1	15.7 ± 2.5	<0.1	10.5 ± 1.1	<0.1	20.5 ± 0.6	<0.1	20.5 ± 0.6	<0.1	20.5 ± 0.6	<0.1						
Aspartate	50.6 ± 7.2	<0.1	37.7 ± 7.5	<0.1	21.0 ± 1.6	<0.1	6.8 ± 2.1	<0.1	96.8 ± 5.8	<0.1	96.8 ± 5.8	11.9 ± 1.9						
Glutamate	146.8 ± 13.0	13.4 ± 4.3	103.4 ± 15.3	22.3 ± 11.5	104.2 ± 4.0	<0.1	41.1 ± 2.7	<0.1	41.6 ± 1.1	<0.1	41.6 ± 1.1	<0.1						
Glutamine	40.4 ± 0.9	<0.1	34.2 ± 2.3	<0.1	41.1 ± 2.7	<0.1	12.4 ± 1.1	<0.1	12.4 ± 1.1	<0.1	12.4 ± 1.1	<0.1						
Glycine	10.9 ± 0.8	<0.1	16.0 ± 1.1	<0.1	11.5 ± 1.3	<0.1												

Table 1 continued

Compound	<i>P. pastoris</i>				<i>S. pombe</i>			
	Fast filtration		MeOH quenching		Fast filtration		MeOH quenching	
	EX	SN	EX	SN	EX	SN	EX	SN
Histidine	4.0 ± 0.2	<0.1	4.2 ± 0.6	<0.1	1.1 ± 0.3	<0.1	1.3 ± 0.2	<0.1
Isoleucine	3.8 ± 0.9	ND	5.5 ± 1.3	ND	<0.1	ND	<0.1	ND
Leucine	10.0 ± 0.9	ND	17.7 ± 1.1	ND	1.3 ± 0.5	ND	1.9 ± 0.3	ND
Lysine	134.3 ± 1.9	<0.1	137.0 ± 7.0	<0.1	33.4 ± 4.0	<0.1	30.0 ± 0.6	<0.1
Phenylalanine	6.0 ± 0.9	ND	9.2 ± 1.1	ND	0.5 ± 0.5	ND	1.0 ± 0.3	ND
Serine	55.3 ± 1.7	<0.1	44.5 ± 3.4	<0.1	7.3 ± 0.5	<0.1	6.3 ± 0.6	<0.1
Threonine	13.0 ± 1.1	<0.1	14.0 ± 1.3	<0.1	4.5 ± 0.3	<0.1	6.0 ± 0.5	<0.1
Tyrosine	75.3 ± 2.8	<0.1	58.1 ± 6.6	<0.1	<0.1	<0.1	<0.1	<0.1
Valine	7.7 ± 1.3	ND	9.4 ± 3.2	ND	2.7 ± 0.8	ND	3.5 ± 1.0	ND

Discussion

With few exceptions, the two sampling strategies obviously not caused significant metabolite leakage in the examined yeast strains. The intracellular levels obtained with both approaches, methanol quenching and fast filtration, were similar (Fig. 1).

This underlines that the two approaches permit authentic quantification of intracellular amino acid analysis in different yeasts. As shown by the following calculation the time period required for sampling prior to quenching or extraction, i.e. below 1 s for methanol quenching and 30 s for filtration and washing, does not influence amino acid pools. For this purpose time constants of amino acid pools were estimated, assuming first order kinetics and pseudo-steady state as described by Wittmann et al. (2004). Time constants hereby result from the ratio of pool size to net flux (Table 4). They are typically in the range of minutes to hours and thus far above the required sampling time. Therefore both methods are suitable sampling techniques for quantification of intracellular amino acids in yeast. However, due to the lack of a quenching step, the filtration method might not be suitable for the analysis of metabolites with very low time constants such as phosphorylated intermediary metabolites. It has to be stated that the appropriate choice of the quenching solution during methanol quenching and the washing solution during fast filtration is important to minimize the loss of metabolites. In the present work, 60% buffered methanol was found optimal for quenching, whereas cells responded only slightly to a change in the washing solution during fast filtration. In addition short contact of the cells with the cold methanol solution is important. Suboptimal sampling conditions, however, might be coupled to increased leakage as observed previously (Villas-Boas et al. 2005). Overall, yeast cells revealed a generally higher robustness against the harsh conditions of low temperature, high solvent content or low osmolarity linked to the sampling procedures as compared to bacterial cells (Bolten et al. 2007; Jensen et al. 1999), a characteristic they seem to share with eukaryotic fungi (Nasution et al. 2006; Ruijter and Visser 1996). The significant leakage of selected amino acids, e.g. aspartate and glutamate from *P. pastoris* during methanol quenching or glycine and serine from *Z. bailii* during fast

Table 2 Influence of the quenching solution during sampling into cold methanol for intracellular amino acid analysis in *S. cerevisiae*

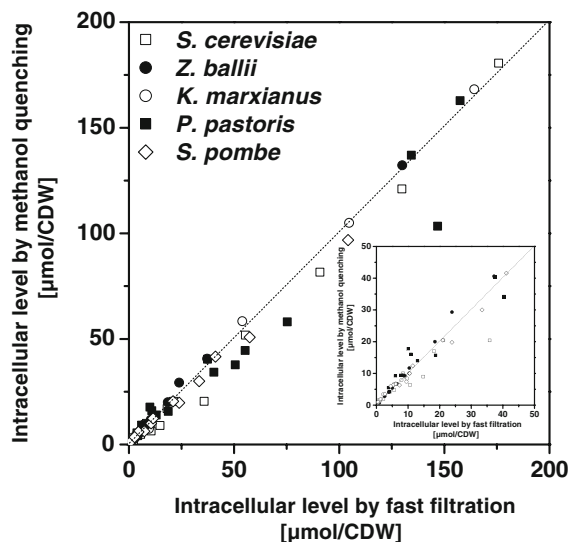
	Quenching with 60% buffered methanol (%)	Quenching with 80% buffered methanol (%)	Quenching with 95% buffered methanol (%)	Quenching with 60% un-buffered methanol (%)
Alanine	96.1 ± 3.4	90.3 ± 2.2	82.9 ± 1.7	94.9 ± 3.6
Arginine	98.3 ± 0.7	95.6 ± 0.6	97.0 ± 1.1	95.8 ± 0.8
Asparagine	>98	>97	>97	>98
Aspartate	94.3 ± 2.3	87.7 ± 1.2	91.6 ± 2.0	90.0 ± 2.6
Glutamate	97.6 ± 1.6	92.8 ± 1.2	94.2 ± 1.1	96.7 ± 1.7
Glutamine	96.7 ± 2.9	91.6 ± 7.6	91.5 ± 1.0	94.1 ± 3.0
Glycine	90.1 ± 3.7	84.5 ± 1.2	74.6 ± 2.4	82.2 ± 3.3
Histidine	97.8 ± 4.8	96.5 ± 1.2	96.4 ± 1.1	93.6 ± 3.7
Lysine	>99	>99	>99	>99
Serine	91.1 ± 4.6	90.6 ± 0.1	88.7 ± 1.2	87.5 ± 4.6
Threonine	94.1 ± 2.1	91.9 ± 0.9	90.2 ± 0.9	91.5 ± 2.1

Hereby, the relative retention of intracellular amino acids within the cell during the quenching step is shown. The value is given in percent. Methanol in different concentration was either buffered with 10 mM HEPES or un-buffered. In each experiment sampling was carried out fourfold in parallel. Cysteine, methionine and tryptophan were below the detection limit in all samples. Leucine, isoleucine, phenylalanine and valine could not be quantified (ND) in supernatant because of interfering ammonia contained in the medium during the HPLC analysis and are therefore not considered here

Table 3 Influence of the washing solution during sampling via fast filtration for intracellular amino acid analysis in *S. cerevisiae*

Compound	Washing with water (%)	Washing with 0.9% NaCl (%)	Washing with fresh medium (%)
Alanine	96.3 ± 3.9	95.3 ± 4.0	92.5 ± 3.5
Arginine	97.1 ± 0.6	96.6 ± 1.0	94.2 ± 0.4
Asparagine	98.5 ± 5.6	98.2 ± 3.4	85.2 ± 6.5
Aspartate	92.9 ± 2.6	92.0 ± 2.2	93.3 ± 3.9
Glutamate	96.7 ± 1.8	95.7 ± 1.8	96.5 ± 1.4
Glutamine	96.4 ± 3.5	96.2 ± 4.0	96.1 ± 3.7
Glycine	90.2 ± 4.1	92.8 ± 5.6	81.1 ± 3.9
Histidine	98.1 ± 5.1	97.6 ± 5.8	93.4 ± 5.2
Lysine	>99	>99	>99
Serine	87.5 ± 5.0	90.1 ± 5.4	91.7 ± 4.8
Threonine	95.0 ± 2.6	94.8 ± 2.5	91.1 ± 1.6

Hereby, the relative retention of intracellular amino acids within the cell during the quenching step is shown. The value is given in percentage of the total pool. The washing solutions applied comprised de-ionized water, 0.9% NaCl and fresh, sterile medium. In each experiment sampling was carried out fourfold in parallel. Cysteine, methionine and tryptophan were below the detection limit in all samples. Leucine, isoleucine, phenylalanine and valine could not be quantified (ND) in supernatant because of interfering ammonia contained in the medium during the HPLC analysis and are therefore not considered here

**Fig. 1** Concentration of intracellular amino acids determined after sampling via fast filtration or methanol quenching in the yeasts *S. cerevisiae*, *K. marxianus*, *P. pastoris*, *S. pombe* and *Z. bailii*. The insert displays the amino acids in the lower concentration range

filtration, however, demonstrate that the adaptation of sampling protocols, previously developed for *S. cerevisiae*, to other yeast strains should be carefully validated.

Table 4 Time constants of intracellular amino acid pools from *S. cerevisiae* ($\mu = 0.22 \text{ h}^{-1}$), *Z. bailii* ($\mu = 0.39 \text{ h}^{-1}$), *K. marxianus* ($\mu = 0.16 \text{ h}^{-1}$), *P. pastoris* ($\mu = 0.18 \text{ h}^{-1}$) and *S. pombe* ($\mu = 0.14 \text{ h}^{-1}$)

Compound	Amino acid fraction in biomass ($\mu\text{mol g CDW}^{-1}$)	Time constant (min)				
		<i>S. cerevisiae</i>	<i>Z. bailii</i>	<i>K. marxianus</i>	<i>P. pastoris</i>	<i>S. pombe</i>
Alanine	360	42.0	15.9	261.3	34.6	28.5
Arginine	142	174.4	11.3	433.6	369.7	173.2
Aspartate/asparagine	342	35.2	12.5	12.7	67.5	39.5
Glutamate/glutamine	570	146.2	40.1	104.3	109.5	109.2
Glycine	328	12.3	3.0	9.0	11.1	15.0
Histidine	71	40.7	5.6	28.0	18.8	6.6
Leucine	295	2.0	0.5	2.7	11.3	1.9
Serine	196	12.5	8.2	18.6	94.0	16.0
Threonine	205	24.2	6.2	11.2	21.1	9.4
Valine	270	5.7	2.8	13.2	9.5	4.3

Time constants were calculated from the measured pool size (Table 1), the specific growth rate and the content of each amino acid in the biomass as described previously (Wittmann et al. 2004). The cellular amino acid content was taken from a previous study with *S. cerevisiae* (Lange and Heijnen 2001)

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