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The Sampling and Sample Preparation Problem in Microbial Metabolomics

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1.1
Introduction

The reproducibility and accuracy of analysis methods has expanded rapidly over the past years. Hyphenated methods, for example, GC-MS and LC-MS/MS (liquid chromatography-mass spectrometry), are now almost routinely used for (semi-) quantitative analysis of tens to hundreds of metabolites in biological samples. It should be realized, however, that the quality of the applied analytical techniques alone is not sufficient to obtain meaningful results. Other important aspects, which are often overlooked, are the methods that are applied to withdraw the samples from a microbial cultivation and the subsequent sample processing procedures. Immediately after withdrawal of the samples, the metabolism should be arrested to preserve the metabolic snapshot represented by the sample by preventing further metabolic conversions. Subsequently, an appropriate extraction method should be applied, which guarantees complete and unbiased release of all metabolites from the cells without significant degradation and inactivation of enzymatic activity. Finally, an accurate and high throughput analytical platform is required for metabolite quantification. In this chapter, we focus on the essential requirements of the sampling and sample processing procedures as well as the methods to validate if these requirements are met in practice.

1.2
Microorganisms and Their Properties

Microorganisms show very large differences in properties, for example, size, structure, degree of complexity, heterogeneity, physiology, nutrient requirements, and so on. These aspects should be considered beforehand because they can be important for the choice of the sampling and sample processing methods to be used. They also determine the meaning of the obtained results, for example, the interpretation of metabolite measurements in cell extracts of prokaryotic microorganisms, which contain only one compartment (cytosol), is more straightforward compared

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to measurements in cell extracts of eukaryotes. As eukaryotic (micro)organisms contain several compartments (cytosol, mitochondria, peroxisomes, etc.) in which the concentrations of metabolites can be very different, the interpretation of the measurements, which are in fact whole cell averages, can be problematic if the metabolites quantified are present in more than one cellular compartment.

1.3 Sampling Methods

1.3.1 The Need for Rapid Sampling

As quantitative metabolomics aims at obtaining accurate snapshots of intracellular metabolite levels, the withdrawal of samples from the microbial culture under study should be sufficiently fast to prevent significant changes in metabolite levels arising from ongoing metabolic activity. This is particularly important if samples are taken from cultures for which one of the medium components is the growth-limiting nutrient (e.g., chemostat or fed-batch cultivations). If the sampling procedure requires too much time, the small amount of growth-limiting nutrient present in the sample will be rapidly exhausted, which will result in changes in metabolic fluxes and consequently in changes in intracellular metabolite levels. Similarly, if samples are taken from aerobic cultivations, the amount of oxygen dissolved in the liquid phase will be rapidly exhausted because of the consumption of oxygen by the cells combined with the very limited solubility of oxygen in water.

It should be realized that the speed at which the ongoing metabolic activity of the cells in the culture sample occurs, and thus the time of exhaustion of the limiting nutrient and/or the available oxygen in the liquid phase, directly depends on the biomass density and the growth rate of the culture from which the sample is withdrawn. For relatively high density and fast growing cultures, this could happen in a few seconds. Considering the pool sizes of intracellular metabolites, which range from 10^{-3} to 10^{2} $\mu mol\ g^{-1}$ dry cell mass [1–3] and the fluxes through the metabolic network, the apparent turnover times of metabolite pools range from fractions of a second to tens of minutes [4]. This implies that to obtain meaningful results a dedicated rapid sampling system is required, whereby the residence time of the sampled culture broth in the sampling system is in the subsecond range.

Furthermore, the metabolic activity of the cells should be arrested as soon as the sample enters the sampling vial. This has generally been accomplished by introducing the culture broth with sufficient velocity (i.e., as a liquid jet) into a dedicated quenching solution (Section 1.4) such that it is instantaneously mixed. A common approach is to use a cold aqueous solvent mixture, for example, 60% aqueous methanol, to cool down the sample below $-20\,^{\circ}C$, thereby minimizing the enzymatic activity. It has been shown that with this method effective quenching of metabolism is obtained only if the mixing of the sample with the cold aqueous

quenching liquid is instantaneous [5]. Another approach is to combine quenching with extraction, by sampling directly into the extraction solution (e.g., perchloric acid) or by integrating extraction in the sampling mechanism itself [6].

1.3.2 Sampling Systems

An overview of different rapid sampling systems, both manually operated and fully automated, has been provided by Schaedel and Lara [7]. Apart from being sufficiently fast, other important requirements for rapid sampling systems are that the amount of sample withdrawn is reproducible; the dead volume is negligible (to prevent that stagnant liquid residing in the system is mixed up with the sample); and especially for sampling during highly dynamic experiments, the time between subsequent samples is sufficiently small to fully capture the dynamics.

A convenient manually operated rapid sampling system for withdrawal of broth samples from bench scale bioreactors has been described by Lange *et al.* [8]. This system consists of a sampling port, inserted in the wall of the bioreactor, with an internal diameter of 1 mm, which is connected to a tube adapter. Sampling is started by removing the dead volume by flushing into the waste. Subsequently, the sample tube is evacuated and directly thereafter, the sample is withdrawn from the bioreactor, facilitated by the vacuum in the sample vial and a slight overpressure in the bioreactor. The liquid flows and evacuation of the sample tube are controlled by electromagnetic pinch valves operated by a timer, allowing the sample volume to be precisely adjusted, that is, with a standard deviation of less than 2%. The authors reported that with this system, they could withdraw samples of 1 ml from a bioreactor, operated at an overpressure of 0.3 bar, within 0.7 s. The residence time of the sample in the system was below 100 ms. However, with this system, the sampling frequency could not be increased much above 1 sample per 5 s because of the many manual handlings that had to be performed. Therefore Schaefer *et al.* [9] developed a completely automated sampling device, whereby the sampling vials were fixed in transport racks, which were moved by a step engine underneath a continuous jet of culture broth, with a flow rate of 3.3 ml s^{-1}, from a stirred tank bioreactor. In this way, each sampling vial, which contained a cold quenching solution, could be filled within 220 ms resulting in a sampling rate of approximately 4.5 samples per second. This automated sampling device was applied to investigate the intracellular metabolite dynamics of glycolysis in *Escherichia coli* after rapid glucose addition to a glucose-limited steady state culture. A disadvantage of this approach is the large amount of broth that is withdrawn, requiring a relatively large bioreactor.

A different approach to integrated sampling, quenching, and extraction from a bioreactor culture has been published by Schaub *et al.* [6]. Hereby, short time heating of the sample is used as the procedure to quench all metabolic activity, at the same time extracting the metabolites from the cells. This is achieved by using a helical coil heat exchanger that allowed continuous withdrawal of the sample from a bioreactor, whereby the broth was rapidly heated to 95 °C. The helical geometry

was chosen to enhance radial mixing, thus improving the plug-flow characteristics of the system. After extraction, the cell debris can be removed by filtration. This sampling device allows withdrawing approximately 5 samples per second. The method has been applied to the analysis of the growth-rate-dependent *in vivo* dynamics of glycolysis in *E. coli* [10].

1.4 Quenching

1.4.1 Quenching Procedures and Their Properties

Different sampling techniques can be combined with various quenching procedures. An essential property of a quenching procedure is that it should accomplish that all metabolic activity is arrested in the moment of sampling. This can be achieved in various ways, for example, by injecting the sample in a solution with an extreme pH (highly acidic or alkaline), by fast heating and subsequent cooling of the sample (see above), by fast cooling to a temperature below $-20\,^{\circ}C$ or by combining different approaches. Rapid cooling can be achieved by injecting the sample in liquid nitrogen or into a cold quenching solution. Examples are cold perchloric acid ($-20\,^{\circ}C$), 1 M alcoholic KOH 50% (v/v) methanol at $-20\,^{\circ}C$ or cold aqueous 60% (v/v) methanol at $-40\,^{\circ}C$.

An important aspect in deciding which quenching method should be used for a particular case is whether the complete broth sample could be extracted (i.e., cells with the surrounding medium) or whether the cells should be separated from the surrounding medium before extraction. Hereby, it should be realized that in common laboratory-scale bioreactor cultivations, the volume of the surrounding medium is 2 orders of magnitude larger than the total cell volume.

This implies that even low concentrations of metabolites outside the cells could compromise the quantification of the intracellular metabolite levels, if the metabolite measurements are carried out in extracts from total broth samples. This is illustrated by the results of metabolite measurements carried out in glucose-limited chemostats of *E. coli*, of which the results are shown in Table 1.1. As shown in Table 1.1, the measured metabolite concentrations in the culture filtrate are 2–3 orders of magnitude lower than the intracellular concentrations. Nevertheless, if the intracellular and extracellular levels are expressed as amounts per gram biomass dry weight (DW) present, it appears that for some metabolites (in this example malate and pyruvate) the amount present in the extracellular medium can be quite significant (around 50%). Thus, if metabolite measurements are carried out in extracts of total broth samples, the levels of certain metabolites will be significantly overestimated.

In order to separate the cells from the surrounding medium before the metabolite extraction step, a quenching procedure must be used, during which the cell membrane integrity is retained. This clearly excludes quenching procedures employing

Table 1.1 Example of extracellular and intracellular metabolite levels measured in a glucose-limited chemostat of *Escherichia coli* K12 MG1655 [3]. To obtain intracellular concentrations a cellular volume of 2.5 ml g^{-1} of dry weight was used.

	Expressed as concentrations		Expressed as amounts per gram of dry weight		
	Culture filtrate (μM)	Cells (μM)	Culture filtrate (μmol g^{-1}DW)	Cells (μmol g^{-1}DW)	Out/in (–)
Glucose-6P	1.3	568	0.13	1.42	0.09
Pyruvate	5.2	300	0.49	0.75	0.65
Malate	4.0	376	0.38	0.94	0.40
Alanine	2.3	536	0.22	1.34	0.16
Glutamate	16.5	29 880	1.56	74.7	0.02

extremely alkaline or acidic solutions, or short time heating is used to arrest metabolic activity. Rapid freezing in liquid nitrogen is also thought to compromise cell integrity because of the formation of ice crystals.

The most commonly used procedure that enables separation of the cells from the cultivation medium is cold methanol quenching [11], combined with cold centrifugation. This method has been used, for example, in combination with a manual rapid sampling system [8], whereby 1 ml of sample is immediately injected in 5 ml of aqueous 60% methanol at −40 °C. After a cold centrifugation step, whereby the temperature of the quenched sample is kept below −20 °C, and an optional washing step, the obtained cell pellet can be extracted for quantification of intracellular metabolites, without interference of extracellular compounds. It should be noted here that this procedure can only be applied successfully if the metabolites are contained within the cells and do not leak out into the quenching/washing solution.

1.4.2
Validation of the Quenching Procedure and Minimization of Metabolite Leakage

From metabolite measurements carried out in the supernatant after cold centrifugation of the quenched sample, de Koning and van Dam [11] concluded that metabolite leakage during cold methanol quenching of yeast was insignificant. This was later confirmed by other authors [8, 12–14]. Contrary to this, Villas-Bôas *et al.* [15] reported significant leakage of several metabolite classes, especially organic acids and a few amino acids from yeast cells after cold methanol quenching.

Later on a systematic study was performed to trace the fate of a large number of metabolites during cold methanol quenching of yeast culture samples [1]. The authors carried out metabolite measurements in all different sample fractions, that is, total broth, culture filtrate, cell pellet, and quenching solutions. To obtain accurate quantification of metabolites in all sample fractions, isotope dilution mass

spectrometry (IDMS) was applied (Section 1.6). This approach allowed them to check the mass balance for each metabolite, and in this way verify the consistency of the measurements, that is, the total amount of each metabolite, as measured in a total broth sample, should be quantitatively found back in the different sample fractions (cell pellet, culture filtrate, and quenching/washing solution). From the obtained results, the authors concluded that quenching of *Saccharomyces cerevisiae* samples in aqueous 60% methanol at $-40\,^{\circ}C$ induced metabolite leakage to such an extent that for most metabolites the levels are underestimated by at least twofold. By varying the methanol content and quenching temperature, they found the optimal condition for yeast quenching and reported that metabolite leakage is entirely prevented by quenching in 100% methanol at a temperature $\leq -40\,^{\circ}C$, with a sample/quenching solution ratio $\leq 1:5$.

The same approach has been used to quantify metabolite leakage during cold methanol quenching, and subsequently optimize the procedure, for other eukaryotic microorganisms. de Jonge *et al.* [16] found that quenching of *Penicillium chrysogenum* in 60% aqueous methanol at $-40\,^{\circ}C$ also resulted in significant metabolite leakage into the quenching solution. In contrast to what was observed for *S. cerevisiae*, increasing the methanol content of the quenching liquid resulted in increased instead of decreased metabolite leakage. The authors reported that metabolite leakage was minimal if mycelium was quenched in 40% aqueous methanol at a temperature of $-20\,^{\circ}C$. For cold methanol quenching of *Pichia pastoris*, it was observed that the methanol content of the quenching solution was not very critical, and for most metabolites measured, leakage was acceptable for methanol contents between 40 and 100% and quenching temperatures between -27 and $-40\,^{\circ}C$ [17]. However, from a critical evaluation, it was found that quenching in 60% methanol at a temperature of $-27\,^{\circ}C$ resulted in the lowest metabolite leakage.

The above findings illustrate that apparently no single condition exists, which is suitable for the quenching of different eukaryotic microorganisms, possibly because of differences in membrane composition. Consequently, it seems necessary to validate and optimize the quenching procedure for each different organism. Although modifications might be required, cold methanol quenching appears to be applicable for quantitative metabolomics of different eukaryotic microorganisms and can thus be used to separate the cells from the surrounding medium to avoid interference with metabolites present therein.

1.4.3
Quenching Procedure for Determination of Intracellular Metabolites in the Presence of Extracellular Abundance

In some cases, it is important to quantify levels of intracellular metabolites that are present in high concentrations in the surrounding medium. Typical examples are excreted products such as antibiotics or organic acids by high producing industrial strains. In this case, proper quantification of the intracellular levels requires complete removal of the amounts present outside the cells and thus

efficient removal of the extracellular medium before metabolite extraction. This could be attempted by increasing the number of washing steps after cold methanol quenching and centrifugation.

However, for efficient removal of highly abundant extracellular metabolites, repeated steps of resuspension of the quenched cell pellet in a cold washing solution and subsequent cold centrifugation would be required because of the carryover of remaining medium after decantation. This is not only a laborious procedure but could also lead to increased metabolite leakage because of the prolonged exposure to the cold methanol containing quenching/washing solution or to partial cell disruption due to the repeated centrifugation steps. For this reason, Douma *et al.* [18] compared the efficiency of cold centrifugation combined with several washing steps with a procedure whereby cold methanol quenching was combined with rapid cold filtration and subsequent filtration-based washing. They applied these procedures to quantify the metabolites involved in the penicillin biosynthesis pathway, in a high producing strain of *P. chrysogenum*. For several of these metabolites, for example, the side-chain precursor phenylacetic acid (PAA), the end product penicillin G (penG), and several by-products, the amounts present in the extracellular medium are 3–4 orders of magnitude higher than the amounts present inside the cells.

In the centrifugation-based method, removal of the extracellular metabolites from the cells was achieved by means of multiple centrifugation and resuspension steps with the cold quenching solution. The cold filtration method was found to be highly superior to the centrifugation method to determine intracellular amounts of metabolites related to penG biosynthesis and indeed allowed the quantification of compounds of which the extracellular amounts were 3–4 orders of magnitude higher than the intracellular amounts.

1.4.4 Quenching of Bacteria

Also for bacteria, cold methanol quenching has been applied. Bolten and coworkers [19] reported that cold methanol quenching of different gram-positive and gram-negative bacteria resulted in a loss of more than 60% of most metabolites from the cells.

From a thorough validation study of cold methanol quenching for *E. coli* [3], whereby metabolite levels were measured in different sample fractions, it was found that a major part of the intracellular metabolites were lost from the cells and ended up in the quenching solution. The authors observed no positive effect of buffering or increasing the ionic strength of the quenching solution. Therefore, they proposed, an alternative to measure the metabolite levels in the total broth sample as well as in the culture supernatant, whereafter the intracellular levels can be obtained by subtraction. This method was successfully applied to quantify intracellular metabolite levels of *E. coli* grown in an aerobic glucose-limited chemostat at a dilution rate of $0.1\ h^{-1}$ [3] and during short term highly dynamic conditions following a glucose pulse [20, 21].

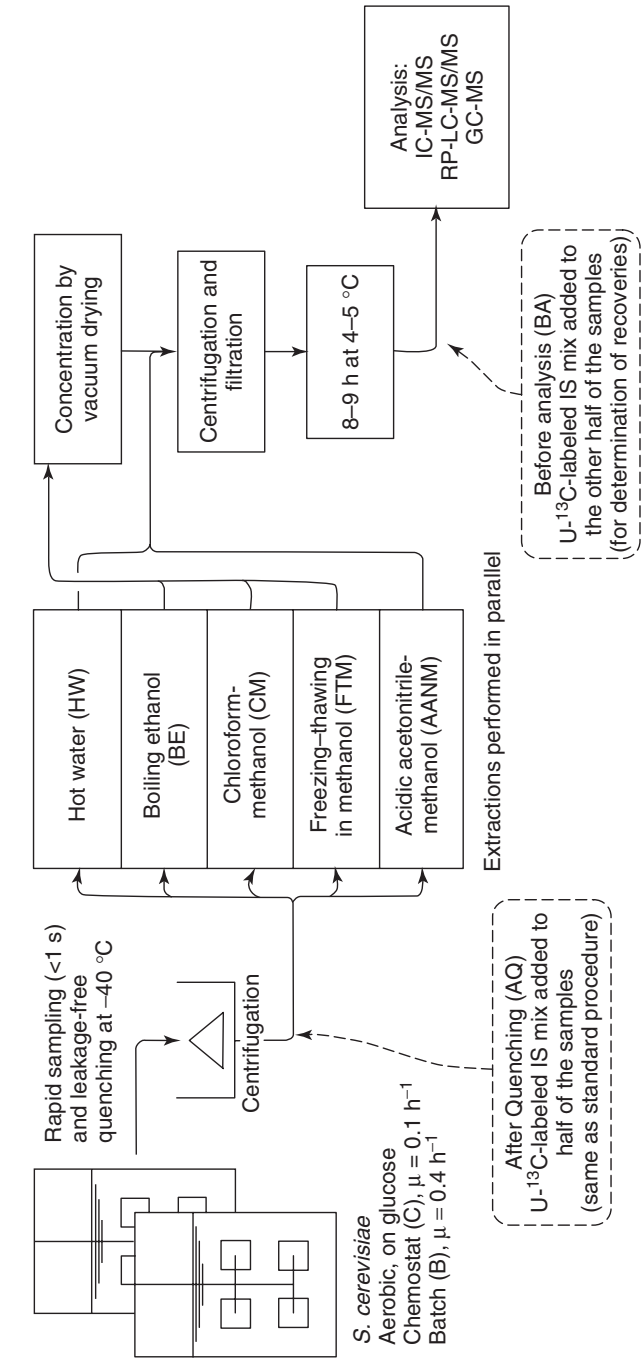

Figure 1.1 Schematic representation of the experimental procedure used for the quantitative evaluation of the applicability of five different extraction procedures for yeast metabolomics. (Source: Figure from Canelas *et al.* [39].) IC, ion-pair LC.

1.5
Metabolite Extraction

1.5.1
Extraction Methods and Their Properties

After obtaining a quenched and washed cell pellet, the metabolites contained within the cells should be extracted completely and quantitatively from them, to allow meaningful quantification. Quite a number of different extraction methods have been described in literature and are based on heating, extreme pH, organic solvents and mechanical disruption, or combinations of these. In earlier works, mainly perchloric acid extraction [22, 23] or hot extraction methods have been applied, for example, short-term boiling in water [24, 25] or aqueous ethanol [26, 27]. More recently, milder methods have been advocated, for example, freeze thaw cycles in cold methanol [28], cold chloroform-methanol (CM) extraction [11], and acidic acetonitrile-methanol (AANM) extraction [29].

Essential requirements that a proper extraction method should fulfill are completeness of extraction, that is, all metabolites should be completely released from the cells; complete denaturation of all enzymes present to prevent further interconversion of metabolites during subsequent sample processing steps; and at the same time, no significant degradation and/or chemical conversion. Finally, the obtained extract should be compatible with the analysis techniques, which will be used for quantification.

Several authors have attempted to evaluate different methods for the extraction of metabolites from different organisms. A summary is given in Table 1.2. It can be seen from this table that the methods that are considered ''good'' or ''best'' by some authors are considered as ''poor'' by others. Clearly, the completeness of extraction might well be different for different microorganisms; however, in some cases, different methods are recommended for the same organism, for example, for *E. coli* (Table 1.2). Important requirements such as the denaturation of enzyme activity and the absence of metabolite degradation should, in principle, not be dependent on the organism used.

1.5.2
Validation of Extraction Methods for Yeast Metabolomics

With the aim to identify the most appropriate extraction method for yeast metabolomics, Canelas *et al.* [39] carried out a quantitative evaluation of the essential requirements, that is, completeness of extraction, enzyme denaturation, and absence of metabolite degradation for five different methods, namely, extraction with hot water (HW), boiling ethanol (BE), CM, freezing-thawing in methanol (FTM), and AANM. The procedure followed is shown schematically in Figure 1.1.

It should be noted that completeness of extraction can never be guaranteed because the exact metabolite contents of the cells cannot be known beforehand.

Table 1.2 Survey of comparisons of extraction procedures for intracellular metabolite analysis in microorganisms.

Source	Microorganism(s)	Authors' evaluation[a], per extraction method[b]							Notes
		PCA	KOH	HW	BE	CM	FTM	AANM	
Bagnara and Finch [30]	*E. coli*	+++	—	+	—	—	—	—	Quantitative, TLC, six metabolites (nucleotides), no recoveries. Tested one other method (TCA).
Lundin and Thore [31]	Five bacteria species	++	+++	+++	+++	—	—	—	Quantitative, enzymatic analysis, three metabolites (ATP, ADP, AMP), recoveries by spiking. Tested six other methods.
Larsson and Olsson [32]	Four algae species	+++	—	++	++	—	—	—	Quantitative, enzymatic analysis, three metabolites (ATP, ADP, AMP), no recoveries. Tested three other methods.
Dekoning and Vandam [11]	*S. cerevisiae*	+++	—	—	—	+++	—	—	Quantitative, enzymatic analysis, up to 13 metabolites (data not shown), recoveries by spiking (only CM)
Gonzalez *et al.* [12]	*S. cerevisiae*	++	—	—	+++	—	—	—	Quantitative, enzymatic analysis, six metabolites, recoveries by spiking
Hajjaj *et al.* [33]	*Monascusruber*	++	++	—	+++	—	—	—	Quantitative, enzymatic analysis, two to six metabolites, recoveries by spiking
Hans *et al.* [13]	*S. cerevisiae*	—	—	+++	+++	—	—	—	Quantitative, HPLC, 17 metabolites (amino acids), recoveries by spiking (only BE)
Maharjan and Ferenci [28]	*E. coli*	+	+	—	++	+	++	—	2D-TLC, semiquantitative for efficacies (total extract intensity and relative intensities for 13 metabolites), no recoveries. Tested one other method (hot methanol)

Jernejc [34]	*Aspergillus. niger*	+++	+++	—	++	—	—	—	Quantitative, enzymatic analysis of three metabolites (organic acids), recoveries assessed by measuring the stability of analytical grade standards during extraction.
Villas-Bôas *et al*. [15]	*S. cerevisiae*	+	+	—	+	++	++	—	GC-MS, qualitative for efficacies (number of peaks detected), quantitative for recoveries, 27 metabolites, by spiking
Wang *et al*. [35]	*E. coli*	—	++	—	+	—	+++	—	Qualitative, ESI-MS (richness and reproducibility of mass spectra), no recoveries
Hiller *et al*. [36]	*E. coli*	+	—	+++	++	—	—	—	Quantitative, enzymatic analysis, seven metabolites, recoveries by standard additions
Rabinowitz and Kimball [29]	*E. coli*	—	—	—	—	+	+	+++	LC-MS, semiquantitative for efficacies (peak heights for nearly 100 metabolites) and recoveries, by spiking (peak heights for 12 metabolites). Tested two other methods (and some variations).
Faijes *et al*. [37]	*Lactobacillus plantarum*	+++	—	—	+++	++	+++	—	Quantitative, enzymatic analysis, three metabolites, recoveries by spiking (only FTM). Tested one other method (chloroform–water)
Winder *et al*. [38]	*E. coli*	+	+	—	+++	+++	+++	—	GC-MS, qualitative (number of peaks detected) and semiquantitative (relative abundance of 21 metabolites) for efficacies, no recoveries

[a] Legend: +, poor/bad; ++, fair; +++, good; underlined, best among the methods tested. Some variations in extraction time, buffers, solvent concentrations and temperatures are considered within the same method.
[b] PCA, perchloric acid; KOH, potassium hydroxide; HW, hot water; BE, boiling ethanol; CM, chloroform–methanol; FTM, freeze-thawing in methanol; AATNM, acidic acetonitrile-methanol; TCA, trichloroacetic acid.
Source: Table from Canelas *et al*. [39].

The only way to determine this is to compare how a number of different extraction methods perform with respect to the amounts of metabolites obtained, under the assumption that at least one of the methods will achieve complete extraction.

The second essential property, denaturation of enzymatic activity, can be evaluated by monitoring whether any interconversion of metabolites takes place after the extract has been obtained, that is, during the subsequent sample processing steps. The third requirement, absence of metabolite degradation, can be evaluated by quantifying the metabolite losses during extraction.

Canelas *et al.* [39] evaluated both metabolite conversion as well as degradation by comparing the obtained metabolite levels after extraction of the same sample in the presence of fully U-^{13}C metabolite mixture with the levels obtained after extraction in the absence of the U-^{13}C mixture (see also the Section 1.6). In the latter case, the U-^{13}C mixture was added after extraction, thus allowing analysis with IDMS. With this procedure, each metabolite is quantified relative to a U-^{13}C-labeled standard, thus correcting for analytical artifacts, for example, sample matrix effects. If the U-^{13}C-labeled standard mix is added before extraction, partial metabolite degradation can also be effectively corrected for because the ^{12}C and U-^{13}C labeled forms of each metabolite are chemically identical and therefore their ratio will not change if partial degradation occurs [40, 41]. This procedure effectively corrects for partial degradation of metabolites during the extraction procedure.

By comparing the metabolite levels in cell extracts obtained in the presence and absence of the U-^{13}C standard mix, the overall process recovery can be quantified for each metabolite analyzed.

$$\text{recovery}_{x,i} = \frac{[x]_i^{\text{BA}}}{[x]_i^{\text{AQ}}} \tag{1.1}$$

where the superscript BA indicates that the U-^{13}C standard mix was added before analysis, that is, extraction was carried out in the absence of internal standards (ISs), and AQ indicates that the standard mix was added directly after quenching, that is, the extraction was carried out in the presence of ISs.

This recovery includes nonspecific losses sample handling, metabolite degradation as well as (inter)conversion. Furthermore, to take into account the time that the samples have to stay in a cooled auto sampler before analysis, the extracts were incubated for 8–9 h at a temperature of 4–5 °C. If only nonspecific losses would occur, a very similar recovery for all metabolites with a value above 90% would be expected. Significantly lower recoveries would be an indication for metabolite degradation, whereas if recoveries well above as well as well below 100% would occur, this would strongly indicate the occurrence of interconversion of metabolites because of the remaining enzymatic activity.

Canelas *et al.* determined the overall process recoveries for the extraction methods shown in Figure 1.1 for 44 metabolites in cell samples of *S. cerevisiae* grown in two different ways, namely in glucose-limited chemostats and in unlimited batch cultures on glucose [39]. The results are shown in Figure 1.2. It can be seen from this figure that the overall process recoveries for the first three extraction methods (HW, BE, and CM) are close to one for the majority of the quantified metabolites,

whereby BE shows the flattest profile. For the other two methods (FTM and AANM), however, large deviations were observed. In case of the FTM method, recoveries were found which were either much higher or much lower than one. This shows that interconversion of metabolites has occurred. It should be noted that the FTM method (freeze-thawing cycles in 50% (v/v) aqueous methanol) is a mild extraction method that will cause cell disruption and release of metabolites but is unlikely to result in complete denaturation of enzymes.

The AANM method did not result in recoveries above 100%, and hence no indications were obtained for interconversion of metabolites. However, especially for the large metabolites (i.e., on the basis of a larger mw), the recoveries were poor (Figure 1.2). The reason for this is not clear. Also, this method seems to be relatively mild because it is carried out at a low temperature and the time of exposure to acidic conditions is not extremely long (45–60 min). From the observation that especially polar charged metabolites exhibit low recoveries, Canelas *et al.* [39] suggested that these could have been caused by poor solubility of these compounds in the cold solvent-rich mixture. If this results in precipitation, a part would be lost during the centrifugation and filtration steps of the sample processing procedure. For further details on the evaluation of these five extraction methods for yeast metabolomics, we refer to the paper of Canelas *et al.* [39]. The final conclusion of these authors was that, at least for yeast, from the five methods tested, BE and CM should be considered as the most reliable methods in terms of completeness of extraction, prevention of metabolite conversion, and metabolite stability.

1.6 Application of ^{13}C-Labeled Internal Standards

Finally, the determination of the metabolites in the obtained cell extracts can be performed with different analytical techniques. Before the application of mass spectrometry for quantification of compounds became common practice, mainly enzymatic assays and chromatographic methods, for example, high-performance liquid chromatography (HPLC), were applied. The advantage of enzymatic assays is their specificity. Their disadvantage is that for each metabolite a different assay has to be used, which makes the analysis of a large number of metabolites very laborious while the amount of sample required is relatively large.

At present, high-throughput hyphenated mass spectrometric techniques, whereby liquid- or gas-chromatographic separation is coupled to mass spectrometric detection, are routinely used for the measurement of small molecules. These methods allow the simultaneous identification and quantification of a large number of metabolites with high selectivity, adequate sensitivity, and minimal sample use (e.g., 5–10 µl for the quantification of several tens of compounds versus 100 µl or more for the enzymatic assay of a single metabolite).

In particular, liquid chromatography/electrospray ionization mass spectrometry (LC–ESI-MS) is nowadays widely used for the quantification of phosphorylated carbon compounds, which are intermediates of primary metabolic pathways [42,

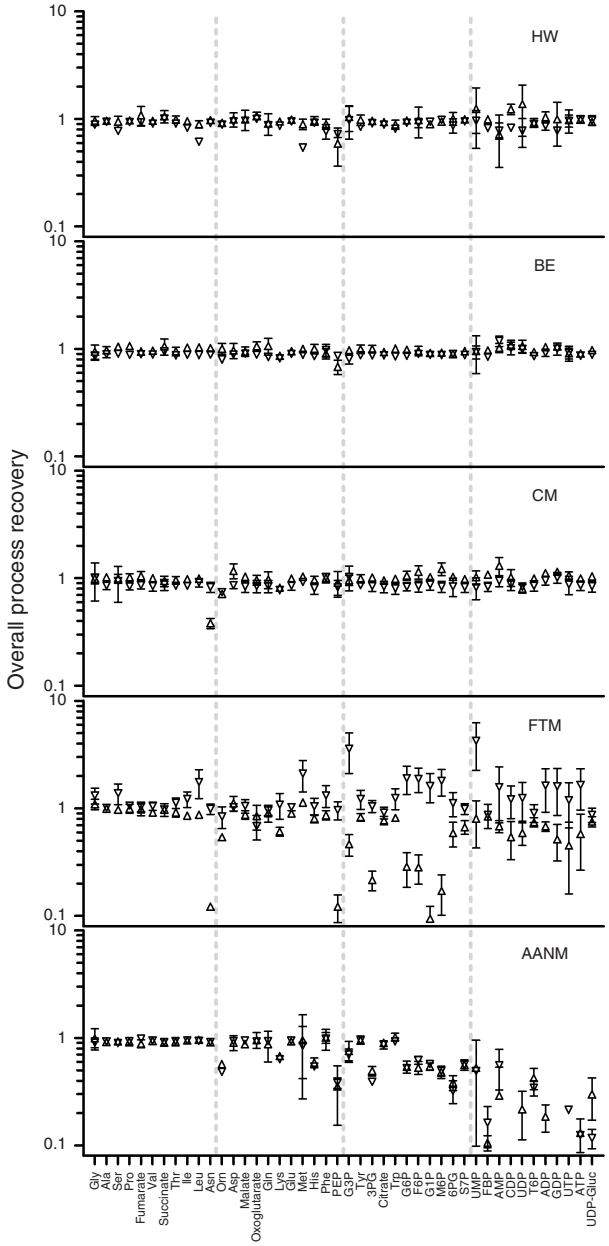

Figure 1.2 Overall process recoveries (calculated from Eq. (1.1)) for 44 metabolites analyzed, in the order of increasing molecular weight, for each of the extraction methods, under two growth conditions (glucose limited chemostat and batch cultivation). Data are averages and standard deviations of duplicate samples each analyzed twice. Legend: ∇, chemostat; Δ, batch. Dashed vertical lines are for guidance only. (Source: Figure from Canelas *et al.* [39].)

43]. However, the performance of LC–ESI-MS can be compromised by ion suppression effects [44] that result in a changing signal response of the analyte, owing to changes in the sample matrix. The composition of the sample matrix is influenced by, for example, the applied quenching and extraction procedures, the species of microorganism used, and the composition of the cultivation medium.

This drawback of LC–ESI-MS quantification can be tackled by applying the isotope dilution technique, whereby for each analyte a stable isotope analog (i.e., isotopolog) is used as an IS in the MS analysis [45]. This technique has found a large variety of applications, as reviewed by Baillie [46]. Because of the high physical-chemical similarities between the labeled IS and the analyte, degradation during sample preparation, instrument drift, and ion suppression effects in LC–ESI-MS can be compensated for. This technique has been widely applied to different research areas, for example, pharmacological, medical studies [46–48]. Also, for microbial metabolomics, it has been shown that the application of IDMS is indispensible to accomplish accurate and reproducible quantification of metabolites [40, 41, 49, 50].

To apply this method, an isotopolog has to be included as IS for each metabolite to be analyzed. Because these isotopologs are not commercially available for most metabolites, they must be produced by cultivating microorganisms on 100% U-^{13}C labeled medium and subsequent extraction to obtain a cell extract containing a 100% U-^{13}C labeled metabolite mixture. If this cell extract is added to each sample before metabolite extraction, partial degradation of metabolites, losses during sample handling as well as instrument drift, and ion suppression effects during LC-MS analysis can be effectively corrected for.

A suitable procedure for the production of the ^{13}C extract is (small-scale) fed-batch cultivation [41]. The advantage of this cultivation method is that the growth rate of the culture can be set to a desired value by controlling the medium feed rate. Cultivation of cells at a certain growth rate can be relevant, for example, to obtain certain desired metabolite levels. Examples are producing strains for which product formation only occurs within a particular range of growth rates (e.g., a high producing strain of *P. chrysogenum* for which the rate of penicillin production, and consequently the intracellular concentrations of the intermediates of the biosynthesis pathway, is highest at a specific growth rate of around 0.03 h^{-1} [51]). Another example is baker's yeast (*S. cerevisiae*) that should be cultivated below the so-called critical growth rate ($\sim$0.3 h^{-1}) because otherwise massive formation of ethanol occurs, resulting in a much lower biomass yield on the supplied U-^{13}C glucose and thus much lower concentrations of U-^{13}C labeled metabolites in the obtained cell extract.

Harvesting of the U-^{13}C-labeled cells can be performed by quenching portions of, for example, 100 ml of broth in 500 ml of cold ($-40\,^{\circ}$C) aqueous methanol followed by extraction in 75% aqueous BE, as described for the production of U-^{13}C yeast extract [41].

Metabolite measurement with IDMS, whereby U-^{13}C labeled cell extract is used as IS, requires the addition of an amount of ^{13}C extract to the quenched cell

samples before extraction. To enable proper quantification, a calibration line has to be made, whereby a fixed amount of the ^{13}C extract is added to different dilutions of an unlabeled standard mixture of the metabolites to be quantified. LC-MS analysis of the calibration mixtures yields a calibration line, whereby for each compound the $^{12}C/^{13}C$ peak area ratio is plotted against the (known) concentration of the unlabeled metabolite in the different dilutions of the standard mixtures. If the volume fraction of the ^{13}C extract in the calibration mixture is identical to the volume ratio of the ^{13}C extract added to each samples versus the volume of the final cell extract, calibration line can be directly applied to convert the measured $^{12}C/^{13}C$ peak area ratio's to the concentrations of unlabeled metabolites in the samples.

It should be noted that partial degradation of metabolites during extraction and/or matrix and ion suppression effects will not result in changes in the $^{12}C/^{13}C$ metabolite ratios because chemical degradation, matrix and ion suppression effects of labeled and unlabeled metabolite analogs should be the same (isotopologs are chemically identical). Also, volume losses due to sample handling will not affect the $^{12}C/^{13}C$ ratio. Therefore, these issues will not affect the outcome of the quantification. Examples of calibration lines for the adenosine nucleotides AMP, ADP, and ATP are shown in Figure 1.3. It can be clearly seen from this figure that the constructed calibration lines based on peak areas of unlabeled standards were

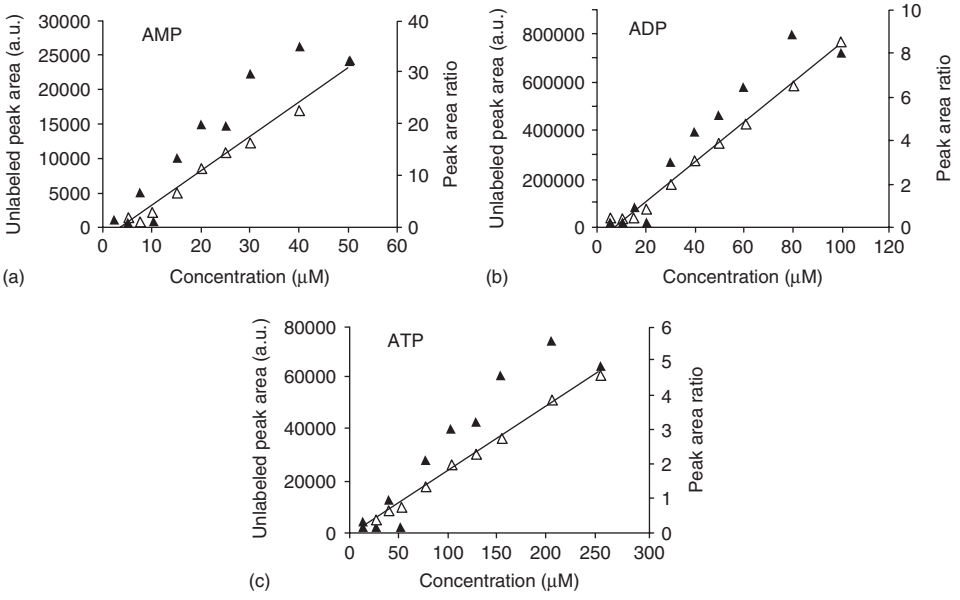

Figure 1.3 Comparison of obtained calibration curves based on measured peak areas of unlabeled nucleotide standards (closed triangles) and peak area ratios of unlabeled to labeled internal standards (open triangles) for monophosphate (AMP), diphosphate (ADP), and triphosphate (ATP) adenosine nucleotides. a.u., arbitrary units. (Source: Figure from Seifar *et al.* [52].)

highly nonlinear compared with the calibration lines based on the ID approach. This shows that the application of U-^{13}C-labeled isotopologs as ISs improves the quality of MS-based metabolome quantification by correcting for partial losses during sample preparation as well as through improvement of the precision of the LC-EI-MS based analytics. It should be realized that enzymatic (inter)conversions of metabolites in the presence of ^{13}C IS mix will result in changes in ^{12}C/^{13}C ratios of the metabolites involved, and thus the application of this standard mix cannot correct for this kind of conversions. The applied extraction procedure should therefore result in complete enzyme denaturation to prevent enzymatic (inter)conversions to occur.

1.7 Conclusions

To obtain meaningful snapshots of the microbial metabolome, rapid sampling and instantaneous quenching of all metabolic activity to prevent further interconversion of metabolites is essential.

An important aspect is the separation of the cells from the surrounding medium, before the extraction procedure, as in many cases intracellular metabolites are also present outside the cells. Although the metabolite concentrations in the extracellular medium are usually low, the much larger volume fraction of the medium in a culture sample, compared to the volume occupied by the cells, could easily result in overestimation of metabolite levels. To avoid this, a quenching procedure should be applied, which does not induce metabolite leakage from the cells and thus allows separation of cells and surrounding liquid after quenching without causing metabolite losses.

So far, no single condition has been found that is suitable for the quenching of different eukaryotic microorganisms; possibly, because of differences in membrane composition. Consequently, it seems necessary to validate and optimize the quenching procedure for each different organism. Although modifications might be required, cold methanol quenching appears to be applicable for quantitative metabolomics of different eukaryotic microorganisms and can thus be used to separate the cells from the surrounding medium to avoid interference with metabolites present therein.

In contrast to this, no suitable quenching procedure has been reported, which allows removal of extracellular metabolites in case of bacterial cultures. Although cold methanol quenching can be applied to bacteria, subsequent cold centrifugation and washing will result in significant loss of metabolites, because of substantial diffusion from the cells into the cold methanol solution, during and after quenching.

The second step after obtaining the cell samples is an extraction procedure to release the metabolites from the cells. Essential requirements that a proper extraction method should fulfill are completeness of extraction, that is, all metabolites should be completely released from the cells; complete denaturation of all enzymes

present to prevent further interconversion of metabolites during subsequent sample processing steps; and at the same time no significant degradation and/or chemical conversion. Several methods have been described in the literature, which do not fulfill these requirements. At present, hot aqueous ethanol boiling seems to be the most common procedure, while HW boiling and CM extraction yield comparable results. These three methods are common in that the enzymes present in the cell samples are effectively denaturated, and therefore enzymatic (inter)conversion of metabolites does not occur.

Metabolite quantification is mostly accomplished with MS-based methods (mainly LC- and GC-MS). This allows the application of IDMS, using stable isotope labeling, for example, ^{13}C, to improve the reproducibility and accuracy of the measurements.

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