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Chapter 2

Fast Sampling of the Cellular Metabolome

Walter M. van Gulik, Andre B. Canelas, Hilal Taymaz-Nikerel, Rutger D. Douma, Lodewijk P. de Jonge, and Joseph J. Heijnen

Abstract

Obtaining meaningful snapshots of the metabolome of microorganisms requires rapid sampling and immediate quenching of all metabolic activity, to prevent any changes in metabolite levels after sampling. Furthermore, a suitable extraction method is required ensuring complete extraction of metabolites from the cells and inactivation of enzymatic activity, with minimal degradation of labile compounds. Finally, a sensitive, high-throughput analysis platform is needed to quantify a large number of metabolites in a small amount of sample. An issue which has often been overlooked in microbial metabolomics is the fact that many intracellular metabolites are also present in significant amounts outside the cells and may interfere with the quantification of the endo metabolome. Attempts to remove the extracellular metabolites with dedicated quenching methods often induce release of intracellular metabolites into the quenching solution. For eukaryotic microorganisms, this release can be minimized by adaptation of the quenching method. For prokaryotic cells, this has not yet been accomplished, so the application of a differential method whereby metabolites are measured in the culture supernatant as well as in total broth samples, to calculate the intracellular levels by subtraction, seems to be the most suitable approach. Here we present an overview of different sampling, quenching, and extraction methods developed for microbial metabolomics, described in the literature. Detailed protocols are provided for rapid sampling, quenching, and extraction, for measurement of metabolites in total broth samples, washed cell samples, and supernatant, to be applied for quantitative metabolomics of both eukaryotic and prokaryotic microorganisms.

Key words Fast sampling, Quenching, Microbial metabolomics, Endometabolome, Exometabolome, Isotope dilution mass spectrometry

1 Introduction

To obtain a systems biology understanding of the behavior of the complex machinery of microbial metabolism and its regulation, it is required to quantitatively study the cells on all different hierarchical levels, e.g., genome, transcriptome, proteome, fluxome, and metabolome, and especially the interactions between them. For some of these levels, the techniques for high-throughput analysis have developed faster than for others. In particular, whole-genome

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sequencing and genome-wide transcriptome analysis have become common practice, while methods for the quantification of intracellular fluxes through metabolite balancing or based on stable isotope (e.g., ^{13}C) labeling have been well established [1]. In contrast to this, quantitative proteome analysis is still far from being a routine technique, although significant progress has been made during the past years [2]. Whole-proteome measurement is not only hampered because the abundance of individual proteins may differ by a factor of a million, but also by the fact that many proteins are subject to posttranslational modifications. It has, for example, been estimated that a single posttranslational modification (N-terminal methionine cleavage, or NME) alters roughly half of the proteins in *Escherichia coli* [3].

Whole-metabolome measurement is hampered by large differences in abundance, structure, and properties of the individual metabolites. Nevertheless, targeted metabolome measurements in microorganisms, mammalian tissues, and plants have already been carried out for more than half a century (*see* ref. 4 and references therein).

1.1 Method Development

1.1.1 Methods for Rapid Sampling and Quenching

It is well known that many metabolites, especially the intermediates of the central metabolic pathways and connected cofactors like ATP and NADH, have turnover times in the order of seconds, as can be calculated from their in vivo pool sizes and conversion rates. This implies that a proper snapshot of the intracellular metabolite levels can only be obtained if sampling and subsequent arrest of metabolic activity are sufficiently fast, that is, significantly faster than the turnover times of the metabolite pools.

Biochemists have been aware of this for many decades as can be inferred from publications from the early 1960s and 1970s, e.g. on the quenching and extraction of rat liver tissue [5–8]. Some of the early works on metabolite measurements in microbial cells already emphasized the importance of arresting all metabolic activity as fast as possible. With the aim to measure the ATP levels in fermentor cultures of *E. coli* under different growth conditions, Cole et al. [9] took 2 mL broth samples from the fermentor directly into ice-cold perchloric acid, achieving simultaneous quenching and extraction. Although the disadvantage of this procedure was that it resulted in a dilution of the sample, this was not a problem in this case because the authors used a sensitive luciferase-based assay for the measurement of the ATP level. Another key disadvantage of combining quenching with extraction of complete culture samples is that no distinction can be made between the metabolites present in the cells and in the supernatant. Partly for this reason, but also because of the relative insensitivity of most of the (in the past mainly enzyme based) metabolite assays, often a separation step has been applied, i.e., filtration or centrifugation, followed by resuspension in a small volume of medium prior to quenching, for example, in cold

perchloric acid (PCA). However, during the delay caused by the concentration procedure, metabolic conversion processes can still proceed, resulting in significant changes in metabolite levels. To avoid this, a rapid filtration method was developed, whereby the filter cake was washed with a cold (−40 °C) 50/50 v/v methanol water solution to quench metabolic activity directly after filtration [10]. Later, de Koning and van Dam [11] proposed a method where sampling from yeast cultures was directly performed into a cold methanol/water mixture (60/40 v/v) of −40 °C without prior filtration. Subsequent separation of cells and supernatant was accomplished by cold centrifugation, thereby including a cold washing step to remove extracellular compounds. This quenching method is currently the most widely applied procedure for eukaryotic cells and in principle allows the measurement of intracellular metabolites without interference of compounds present in the cultivation medium. A schematic representation of this procedure is shown in Fig. 1a.

It has been reported, however, that in case of prokaryotic cells, the application of the cold methanol quenching method results in significant leakage of metabolites into the quenching solution [13, 14]. To quantify metabolite leakage during cold methanol quenching of *E. coli*, Taymaz-Nikerel et al. [15] applied a procedure developed by Canelas et al. [16], whereby metabolite measurements are carried out in all different sample fractions and a mol balance approach is used to track down the fate of the metabolites (*see* Fig. 2). From the results for a few different metabolites, shown in Fig. 3, it can be seen that after quenching and subsequent washing of *E. coli* cells, the major part of the intracellular metabolites is found back in the quenching and washing solutions. Therefore, a differential method, whereby metabolite measurements are performed in total broth samples as well as in the supernatant, to

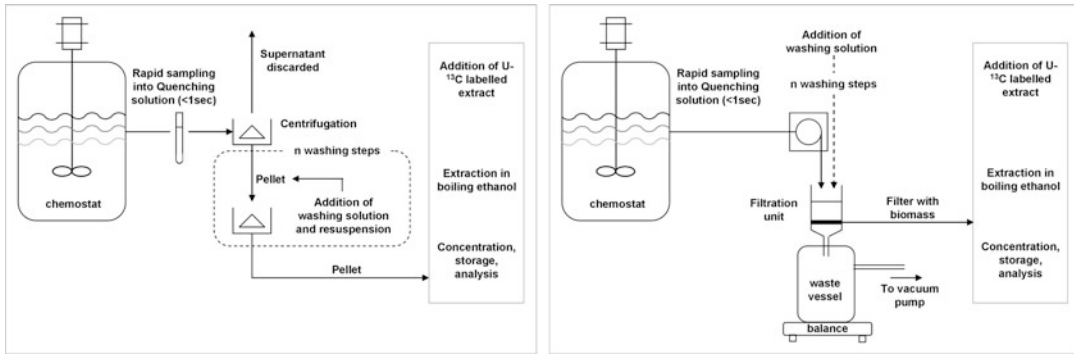

Fig. 1 Schematic overview of two different sampling procedures, *left panel*: rapid sampling and conventional cold methanol quenching combined with cold centrifugation and centrifugation-based washing; *right panel*: rapid sampling and cold methanol quenching combined with cold filtration and filtration-based washing (Figure from Douma et al. [12])

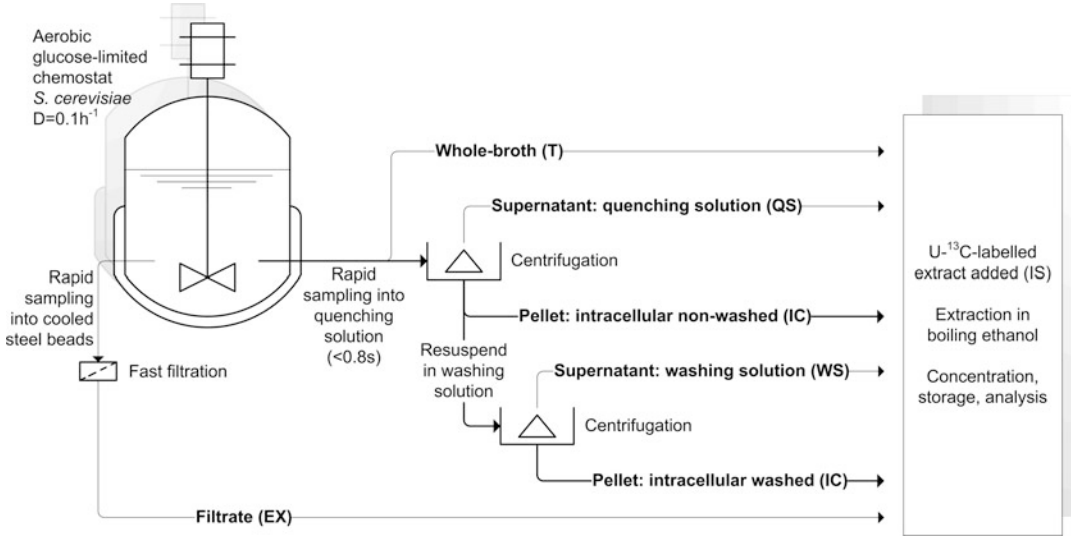

Fig. 2 Measurements carried out in different sample fractions to enable a mass balance-based approach for quantification of metabolite leakage during quenching (Figure from Canelas et al. [16])

obtain the intracellular amounts by subtraction, has been developed and successfully applied for metabolome measurements in *E. coli* [15], *see* Fig. 4.

1.1.2 Fast Sampling Devices

Probably the first attempt of rapid sampling from a laboratory-scale bioreactor has been reported in 1969 by Harrison and Maitra [17]. Sampling was performed via a port in the base plate of the reactor. To remove the broth from the dead volume of the sampling port prior to the withdrawal of the sample, 5 mL of culture was allowed to flow to waste shortly before sampling. The authors measured the sampling time and the subsequent time required to fully mix the sample with the quenching solution, by sampling 9 mL of a 10 M alkali solution into 1 mL of concentrated HCl in a test tube to which a Thymol Blue indicator was added. The sampling procedure was recorded with a cine-camera at 67 frames/s. In this way, they determined that the maximum time interval between the removal of the sample from the culture vessel and coming into contact with the quenching solution in the sample tube was approximately 0.1 s. Subsequent mixing with the quenching solution took about 0.08 s. This rapid sampling method was applied to measure the levels of the adenine nucleotides and some intermediates of central metabolism in chemostat cultures of *Klebsiella aerogenes* under different oxygen supply conditions and as response to substrate pulses.

With the aim to avoid the contamination of the sample with the contents of the dead volume of the sample valve, Iversen [18] constructed a rapid sampling valve wherein the remaining broth was removed from the dead space of the valve after each sampling

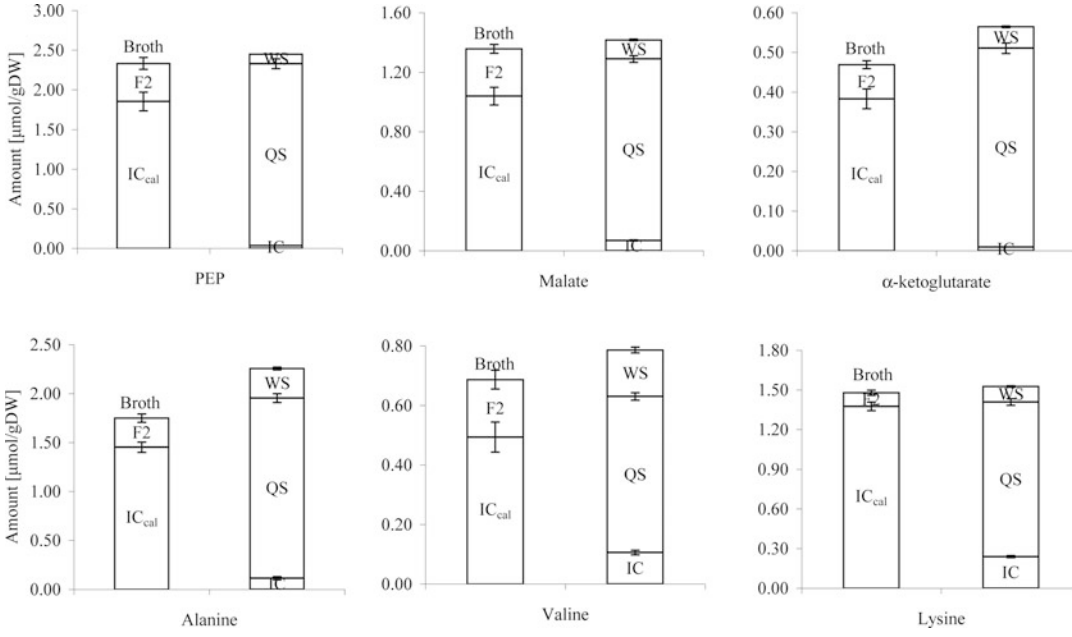

Fig. 3 Examples of results of the balancing approach for quantification of metabolite leakage during the cold methanol quenching procedure: (F_2) amount measured in the filtrate, IC_{cal} ($= B - F_2$) calculated amount in the cell pellet, (WS) measured amount in the washing solution, (QS) measured amount in the quenching solution, (IC) measured amount in the biomass pellet. Bars represent the averages, with their standard errors, of four replicate samples taken from two independent chemostat experiments, analyzed in duplicate (Figure from Taymaz-Nikerel et al. [15])

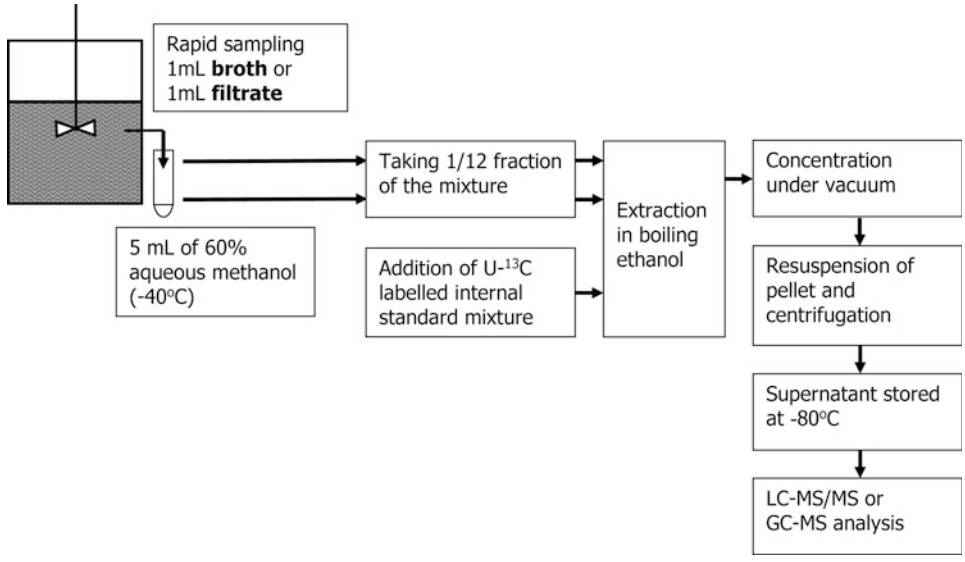

Fig. 4 Workflow of the differential method for intracellular metabolite quantification (Figure from Taymaz-Nikerel et al. [15])

by flushing the valve with a disinfectant, sterile water or sterile air. With the aim to minimize the part of the sample to waste and thus avoiding a too large decrease of the culture volume as a result of sampling, Theobald et al. [19] developed a rapid sampling system with a minimal dead volume. This system consisted of a hypodermic needle inserted into the bioreactor via a silicone membrane, and a sterilizable miniature valve coupled to a HPLC capillary with an internal diameter of 0.7 mm. With this system, the dead volume was only 200μL and could be neglected compared to the total sample volume of approximately 5 mL. The sampling speed was increased by using evacuated sampling tubes. The system allowed concurrent sampling and quenching in less than 0.5 s with a maximum sampling frequency in the order of one sample per 5 s, under aseptic conditions. The system was applied for measuring the in vivo time profiles of the adenine nucleotides in glucose-limited chemostat cultures of yeast during transition from glucose limitation to glucose excess.

A limitation of the above-mentioned sampling systems is that they are manually operated, which is relatively laborious, and that the variation in sample volume depends on the skills of the operator. For these reasons, Larsson and Törnkvist [20] developed a sampling system operated via electrically controlled valves. With this system, samples could be withdrawn within 0.15 s. In between sampling, the remaining liquid was removed from the system by under-pressure. This system was applied for the measurement of the residual glucose concentration in glucose-limited fed-batch fermentations, whereby the samples were quenched and extracted in perchloric acid.

Another example of an electrically operated rapid sampling system has been published by Lange et al. [21], *see* Fig. 5. The system consisted of a sampling port with an internal diameter of 1 mm, connected to a tube adapter. Sampling was started by removing the dead volume contents by flushing to waste. Subsequently, the sample tube was evacuated, and directly thereafter, the sample was withdrawn from the bioreactor. The liquid flows and evacuation of the sample tube were controlled by electromagnetic pinch valves operated by a timer, allowing the sample volume to be precisely adjusted, i.e., with a standard deviation of less than 2%. The total inner volume of the sample system was approximately 100μL, of which 50μL could not be flushed before sampling and should be considered as the dead volume. The authors reported that with this system samples of 1 mL could be withdrawn from a bioreactor, operated at an overpressure of 0.3 bar, in 0.7 s. The residence time of the sample in the system was below 100 ms. The mixing time with the quenching liquid was assumed to be equal to the value measured by Harrison and Maitra [17] which was 80 ms.

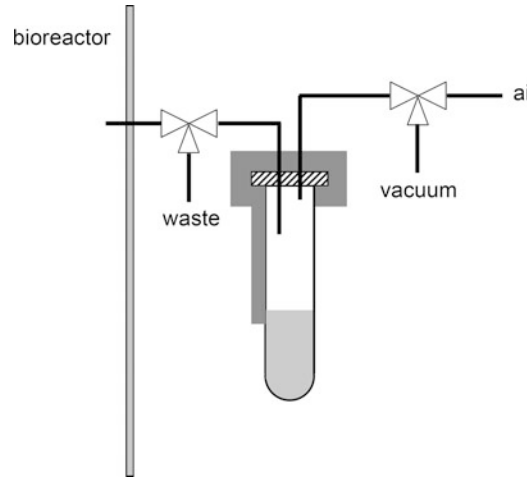

Fig. 5 Schematic representation of the rapid sampling system developed by Lange et al. [21]. The operation procedure is described in the text

Still also with this system the sampling frequency could not be increased much above one sample per 5 s, because of the many manual handlings that had to be performed. Therefore, Schaefer et al. [22] developed a completely automated sampling device, whereby the sampling tubes were fixed in transport racks which were moved by a step engine underneath a continuous jet of sample, with a flow rate of 3.3 mL/s, from a stirred tank bioreactor. In this way, the sampling tubes containing the quenching solution could be filled within 220 ms, resulting in a sampling rate of approximately 4.5 samples per second. This automated rapid sampling device was applied for investigation of the intracellular metabolite dynamics of glycolysis in *Escherichia coli* after rapid glucose addition to a glucose-limited steady state culture.

A completely different approach to increase the sampling frequency was developed by Weuster-Botz et al. [23]. The basic idea was to perform sampling, inactivation of metabolic activity and extraction of intracellular metabolites in a continuous way in a tube, connected to a well-controlled bioreactor. In this way, the highly dynamic metabolite patterns resulting from a sudden disturbance of the culture in the reactor were fixed at a certain position in the sampling tube. The system consisted of a custom-made sampling probe with an inlet of 4 mm diameter, which contained a second inlet for continuous supply of quenching/extraction solution and an outlet of 8 mm diameter connected to the sampling tube. Cold (−40 °C) perchloric acid was used as quenching/extraction solution, which was mixed with the sample 3 mm from the entrance of the sample probe. The sampling tube was made from polyethylene with an internal diameter of 8 mm and a total length of 100 m and was wound up to a coil with a diameter of 0.5 m.

Before sampling, the tube was completely filled with water, to ensure a constant pressure driven flow of sample through the tube. After perturbing the bioreactor culture, continuous sampling was started, and the system was operated in such a way that the complete tube was filled within 200 s. Subsequently, the complete sampling coil was disconnected and immediately frozen at −80 °C. To obtain single samples at different reaction times, the frozen tube was cut into parts with lengths of 0.33 m. In this way, each piece contained an amount of sample representing a time period of 0.64 s. It was demonstrated by the authors that the system could be successfully applied to capture the short time dynamics, on a sub-seconds scale, of some glycolytic intermediates of chemostat-cultivated *Zymomonas mobilis* as a response to a glucose pulse.

More recently, a different approach for integrated sampling and extraction from a bioreactor culture has been proposed by Schaub et al. [24]. Fast heating of the sample was used as procedure for simultaneous quenching and extraction. This was achieved by using a helical coil heat exchanger which allowed continuous withdrawal of sample from a bioreactor followed by rapid heating to 95 °C. The helical geometry was chosen to enhance radial mixing. The residence time of the sample in the device before heating was 200 ms. Thereafter, the sample was heated at 95 °C for 2.5 s which appeared sufficient for complete metabolite extraction. After extraction, the cells were removed by filtration. This sampling device allowed withdrawing five samples of 0.7 mL/s.

A dedicated sampling device, the BioScope, has been developed to carry out pulse response experiments outside the bioreactor [25, 26]. In this device, actually a mini plug flow reactor which can be coupled to any bioreactor, experimentation and sampling are combined. The device has been successfully applied to elucidate short-term metabolite dynamics in different microorganisms [27–30].

To allow fast sampling from fungal cultures, Lameiras et al. [31] constructed a system with which a sample could be withdrawn from an external broth loop connected to a 7-L bench scale bioreactor with a working volume of 4.5 L. Using a fast peristaltic pump, the fungal broth was pumped from the reactor via the sampling device and back into the reactor with a flow rate of 40 mL/s. The internal diameter of the broth loop was 8 mm, which made sure that the formation of fungal pellets would not lead to blockage of the sampling device. An additional advantage of this device is that, due to the continuous flushing with broth, it has no dead volume and that the amount of broth withdrawn from the reactor is limited to the sample itself. The operating principle of the sampling device is shown schematically in Fig. 6.

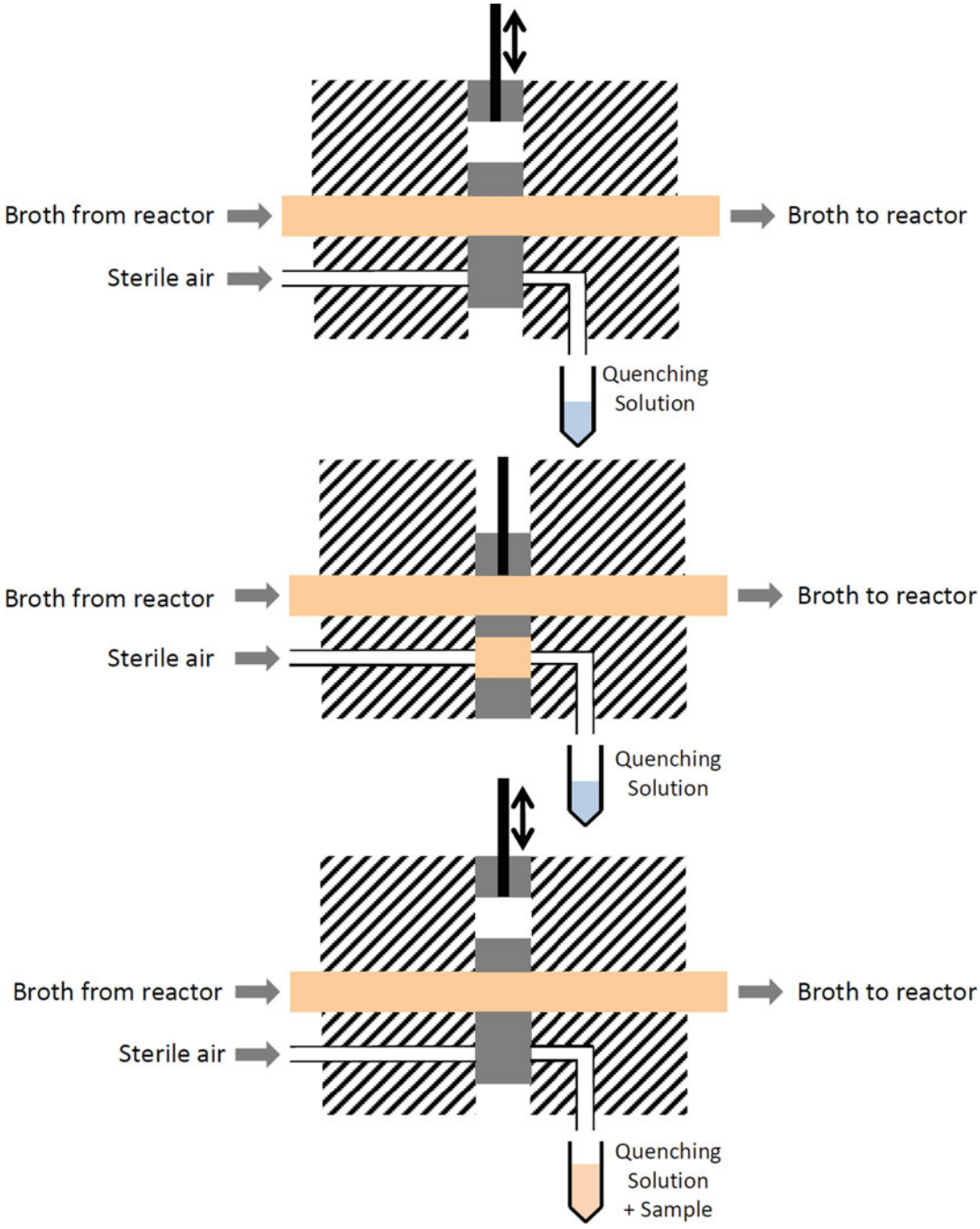

Fig. 6 Operation principle of a rapid sampling device for fungal cultivations (Figure from Lameiras et al. [31])

1.1.3 Methods for Fast Quenching of Metabolism

In the literature on fast sampling methods discussed above, all methods have been applied with certain quenching procedures. It should be realized, however, that many different combinations of

sampling and quenching methods are possible. Among the different quenching protocols developed for microorganisms, two main groups can be recognized, namely procedures which allow separation of cells and supernatant and procedures which do not. Methods wherein quenching and metabolite extraction are combined belong to this last category. Clearly, the methods which do not allow separation of the cells after quenching are only suitable to measure compounds of which the amount present in the supernatant is negligible compared to the intracellular amount. Published data for *S. cerevisiae* and *E. coli* [15, 31] show that most metabolic intermediates are also present, in trace amounts, in the extracellular medium. However, due the fact that the volume fraction of medium is much larger than the volume fraction of cells (roughly a factor 100 in laboratory cultivations), these trace amounts may be sufficient to cause gross overestimation of intracellular pools if they are not removed or properly taken into account.

Separation of cells and surrounding culture medium can be achieved by either filtration or centrifugation. These procedures should be carried out rapidly enough, and preferably at low temperature, to avoid continuation of metabolic activity, otherwise the measured metabolite levels are not representative for the applied cultivation conditions [4].

De Koning and van Dam [11] were the first who combined fast quenching and subsequent separation of cells and supernatant in one procedure, thereby applying cold (−40 °C) 60% aqueous methanol as quenching solution. Their method was inspired by the sampling procedure published by Saez and Lagunas [10] who applied filtration to separate the cells followed by subsequent washing of the filter cake with cold (−40 °C) 50% methanol before freezing the cells in liquid nitrogen.

De Koning and van Dam applied their method for yeast, whereby they directly sprayed 15 mL of culture broth into 60 mL of cold 60% aqueous methanol solution. Separation of cells and surrounding liquid was achieved by cold centrifugation (5 min at 9000 × *g* at −20 °C). The obtained cell pellet was subsequently extracted to release the metabolites.

This method in principle allows determining in vivo intracellular metabolite levels without interference of extracellular metabolites present. However, an important requirement for this method to be applicable is that the cells remain intact and that no metabolite release into the cold methanol solution occurs. This was checked by de Koning and van Dam [11], who verified whether the metabolites which were present in significant amounts in the cells, could also be detected in the culture supernatant and in the supernatants obtained after cold methanol quenching. From the obtained results, the authors concluded that no significant metabolite leakage occurred of the measured intracellular metabolites (glycolytic intermediates, pyruvate, NAD, NADH, and ATP). Later on, other

workers have verified whether metabolite leakage occurred during application of the cold methanol quenching method for different microorganisms. The published results appeared contradictory (*see* Canelas et al. [16] and references therein) most probably because of differences in sensitivity of the applied analytical procedures.

Recently, it has been shown that, especially in case of bacteria, cold methanol quenching induces extensive metabolite leakage, possibly due to the so-called cold shock phenomenon [13–15]. There are indications that also during quenching of yeast culture samples, metabolite leakage occurs, although the current literature is not consistent on this issue [16, 32]. Nevertheless, the cold methanol method is still the most widely used method for rapid quenching of microbial cultures [21, 31, 34, 35]. However, before applying the method, it should be verified for each particular microorganism whether metabolite leakage occurs, and if so, how this can be minimized or avoided [15, 31].

1.1.4 Extraction of Metabolites from Quenched Cell Samples

The next step in the procedure is the extraction of the metabolites from the quenched sample. Ideally, the applied extraction procedure should result in unbiased and complete extraction of all metabolites from the cells, should not lead to conversion and/or degradation of metabolites during extraction and subsequent sample processing, and should be compatible with the analysis methods to be applied. Extraction can be achieved using high temperature, extreme pH, organic solvents, mechanical stress, or combinations of these. Well-known methods which have been employed since the 1950s are extraction in perchloric acid [36, 37], hot water [38, 39], and boiling ethanol/water [40, 41]. More recently, the tendency has been to apply milder extraction methods, to prevent degradation of metabolites as much as possible. In these methods, extraction is carried out at low temperatures, sometimes combined with repeated freezing and thawing to disintegrate the cells. Examples are cold chloroform methanol/extraction [11], freeze-thawing in methanol [42], and cold acetonitrile-methanol extraction [43]. A quantitative evaluation of different extraction methods for application to metabolome analysis of yeast has been published by Canelas et al. [44]. In this study, the addition of ^{13}C-labeled internal standards at different stages of sample processing has been applied to determine the metabolite recoveries. Canelas et al. concluded that the boiling ethanol/water and chloroform/methanol extraction methods performed best, in terms of efficacy and metabolite recoveries. Application of methods which do not ensure complete enzyme inactivation, e.g., freeze-thawing in methanol, significantly affected the outcome of the metabolome measurements, due to enzymatic conversion of metabolites in the samples. Metabolite recoveries upon extraction of yeast cells with acidic acetonitrile-methanol appeared low for larger and more polar metabolites (*see* Fig. 7).

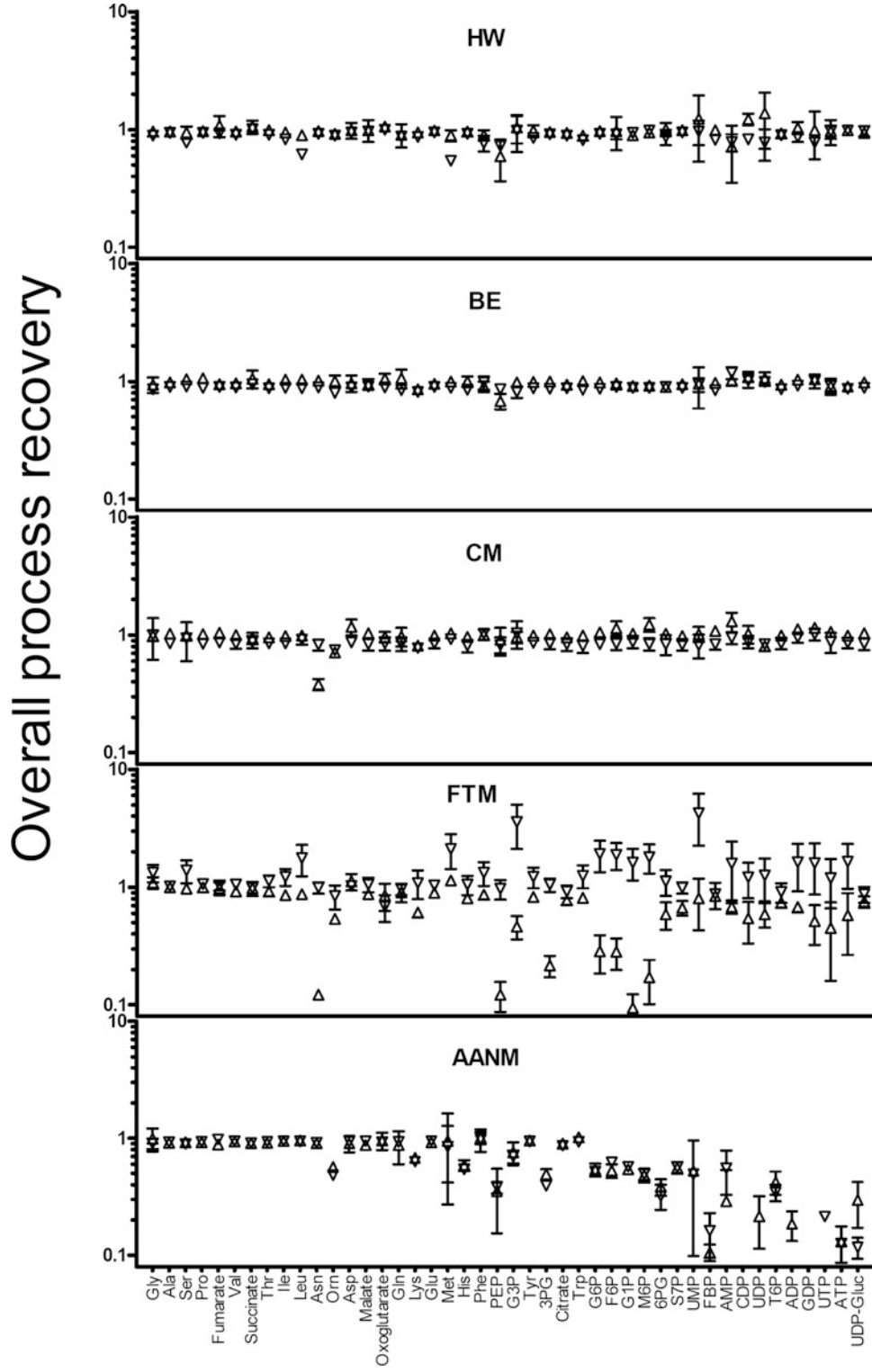

Fig. 7 Overall process recoveries for 44 metabolites analyzed in yeast, in order of increasing molecular weight, for each of the extraction methods, under two growth conditions, chemostat and batch cultivation. Data are averages and standard deviations of duplicate samples each analyzed twice. Legend: ∇, chemostat; Δ, batch (Figure from Canelas et al. [44])

1.1.5 Analytical Procedures

Finally, high-throughput analysis methods are required for selective and precise quantification of a large variety of metabolites. In the past almost exclusively enzyme based methods have been used [45] which have the advantage that they are very specific for a particular metabolite, but the disadvantage that for each metabolite a different assay is required and that some of the enzymes needed might not be commercially available. With the improvement of GC and HPLC techniques, these have therefore increasingly been used. During the last decade, sensitive high-throughput mass spectrometry-based methods (mainly GC-MS and LC-MS/MS) have enabled the measurement of large numbers of different metabolites in a small amount of sample. Especially with the application of U-^{13}C-labeled internal standards, enabling to perform isotope dilution mass spectrometry (IDMS), the precision of MS-based metabolome measurements has increased significantly [46, 48].

2 Materials

2.1 Cold Methanol Quenching Combined with Cold Centrifugation

1. Rapid sampling setup (*see* **Note 1**), e.g., the system published by Lange et al. (for a complete description, *see* ref. 21).
2. Cryostat, filled with a suitable cryo liquid (e.g., ethylene glycol) and capable of reaching a temperature of −40 °C.
3. 60% (v/v) methanol/water mixture.
4. Appropriate test tubes (e.g., polypropylene (PP) tubes of 14 mL, 17 mm diameter) with caps.
5. Cooled laboratory centrifuge capable of reaching a temperature of at least −20 °C.
6. A −40 °C freezer to pre-cool the centrifuge rotor.

Precautions:

Methanol and ethylene glycol (the most commonly used cooling fluid) are toxic substances. Always wear (impermeable) gloves and safety goggles when manipulating the samples, and avoid contaminating surfaces and equipment.

2.2 Additional Materials for Cold Methanol Quenching Combined with Cold Filtration

1. For fungal cultures: glass fiber filters (e.g., type A/E, Pall Corporation, East Hills, NY, USA, 47 mm diameter, 1μm pore size). For yeast and bacterial cultures: Hydrophilic polyethersulfone (PES) membrane filter with a pore size of 0.2–0.45μm (e.g., Supor, Pall, USA).
2. Peristaltic pump capable of reaching a flow rate of at least 300 mL/min.
3. Filtration setup with vacuum pump.
4. Balance.
5. Water bath at 70 °C.

6. 50-mL test tubes with screw cap.
7. Syringe filters with a pore size of 0.2μm filters (e.g., FP30/0.2 CA-S; Whatman, Maidstone, England).

Note: The bioreactor from which the samples are taken should be equipped with a sampling port connected to tubing which runs through the peristaltic pump (*see* Fig. 1, right panel).

2.3 Rapid Sampling of Culture Filtrate

1. Plastic syringes with a volume of 10, 30, or 60 mL (depending on the sample volume required).
2. Stainless steel beads with a diameter of 4 mm.
3. Syringe filters with a pore size of 0.45μm, e.g., Milex HV (Millipore, Cork, UK).

2.4 Extraction

1. 75% (v/v) ethanol/water mixture.
2. If isotope dilution mass spectrometry (IDMS) is used for metabolome analysis (*see* **Note 2**): U-^{13}C-labeled cell extract containing all metabolites which have to be measured, in sufficient amounts (*see* ref. 45).
3. Vacuum evaporation system (e.g., RapidVap (Labconco Corporation, Kansas City, MI)).
4. 0.2-μm Durapore PVDF centrifuge filters.

3 Methods

It has been found that in microbial cultivations, a large part of the cellular metabolome is also present in the cultivation medium. This is partly a result of cell lysis, but presumably also due to the structure of the cell membranes and the transport proteins located in them, which permit metabolites to diffuse into the medium. Metabolome analysis of microbial cultures may therefore include, apart from the measurement of the intracellular metabolite levels (the endometabolome), also the measurement of the extracellular levels (the exometabolome). Below we will present fast sampling methods for both the endo- and the exometabolome.

3.1 Rapid Sampling for Endometabolome Analysis: Cold Centrifugation Method

This protocol is typically suited for rapid sampling of microorganisms which show negligible leakage of metabolites into the quenching solution. Because this method includes a centrifugation and a washing step, the metabolites present in the cultivation medium are removed. This allows proper quantification of the intracellular metabolites without interference of the exometabolome. It should be noted that although the concentrations of metabolites in the medium are usually much lower than within the cells, the amount of extracellular metabolites in a broth sample can still be significant

compared to the intracellular amount, because in most laboratory cultivations, the volume of the supernatant is roughly two orders of magnitude larger than the volume of cells. In the protocol below, a sample volume of 1 mL is assumed. The method is, however, easily scalable to smaller or larger sample volumes. *See* Fig. 1, left panel, for a schematic overview of the procedure.

3.1.1 Preparation

It is advisable to carry out the following preparatory steps the day before the sampling is performed:

1. For n samples, prepare:
   - *n* test tubes containing 5 ml of 60% v/v MeOH, for sampling. Number and weigh them. Close all tubes with caps and store at −40 °C.
   - *n* test tubes containing 5 ml of 60% v/v MeOH for the washing step. Close tubes and store also at −40 °C.
   - *n* test tubes containing 5 ml of 75% v/v EtOH (68% m/m) for the extraction step. Close tubes and store in the fridge.
2. Set the temperature of the centrifuge to −20 °C and put the appropriate centrifuge rotor in a −40 °C freezer. Turn on the cryostat and set the temperature to −40 °C.
3. Connect the rapid sampling setup to the bioreactor to be sampled.

The next steps are best performed on the same day the sampling is performed:

1. If IDMS analysis is used (*see* **Note 2**): Let the frozen ^{13}C-labeled extract thaw in the fridge. Make sure that you use the same uniform solution for all samples and standards. Keep the vial containing the ^{13}C extract closed and cold, e.g., on ice.
2. Place the tubes containing 60% (v/v) methanol, required for sampling and washing of the cell pellet, in the cryostat at −40 °C.
3. Adjust the timer controlling the electronic valve(s) of the rapid sampling system such that the weight of the sample taken equals 1.00 ± 0.05 g.
4. Calibrate the pipette required for ^{13}C extract additions (typically 100μL).
5. Switch on a suitable water bath and let it reach a temperature of 95 °C before sampling is started.
6. Place the tubes containing the 75% ethanol next to the water bath and allow them to warm up to room temperature.

3.1.2 Sampling

1. Withdraw 1.0 mL of broth into a sampling tube (containing 5 mL of 60% methanol at −40 °C) using the rapid sampling device, mix directly after sampling by vortexing. Close with a cap and place the tube back in the cryostat. Repeat until the required number of samples has been taken.
2. Weigh each tube for exact sample amount determination (by subtracting the weight of the tube containing the 5 mL of 60% methanol determined the day before) and put back in the cryostat. Make sure the cryostat fluid (e.g., ethylene glycol) is effectively wiped from the walls of the tube as it can affect the weighing and lead to overestimation to sample weight (especially with small sample volumes), *see* **Note 3**. The weighing procedure should be expeditious to prevent warming up of the sample (*see* **Note 1**).
3. Remove the centrifuge rotor from the −40 °C freezer and put back into the cooled centrifuge. Centrifuge the quenched sample, e.g., at 2000 × *g* for 5 min. Centrifugation conditions should ensure a stable pellet which can still be resuspended. It might be required to adapt the centrifugation speed for a particular microorganism and/or cultivation condition.
4. Decant and discard the supernatant and resuspend the cell pellet immediately by adding 5 ml of 60% (v/v) methanol of −40 °C and rapid vortexing.
5. Centrifuge again, decant, discard the supernatant, and place the tube back in the cryostat.
6. If IDMS is used for metabolite quantification: add ^{13}C extract (typically 100μL) to each washed cell pellet (*see* **Note 2**).

Note: From sampling to decanting, the samples should be exposed to methanol as short as possible to minimize leakage of metabolites from the cells in the quenching solution (*see* **Note 4**).

3.1.3 Extraction of the Cell Pellets

Boiling ethanol/water extraction is applied to release the metabolites from the cell pellets (*see* **Note 5**). During this procedure, each tube with the extraction solution (75% v/v EtOH) is heated (e.g., 4 min for a volume of 5 mL) to reach a temperature of 95 °C. Thereafter, the hot ethanol solution is transferred to tubes containing the cell pellets. After resuspension of the cells in the hot ethanol solution, they are kept at 95 °C for a period of 3 min. This procedure effectively releases all metabolites from the cells and, at the same time, results in denaturation of the enzymes present, which prevents further (enzyme-catalyzed) conversion of metabolites in the samples (*see* **Note 5**).

1. Remove the required number of tubes containing 5 ml of 75% v/v EtOH from the fridge.

2. Put the tubes containing 5 ml of 75% v/v EtOH in the 95 °C water bath to heat up with convenient time intervals (e.g., 30 s).
3. After 4 min, transfer (by pouring) the hot ethanol solution of the first tube to the tube containing the first cell pellet, rapidly resuspend the cells by vortexing and put back in the hot bath. Make sure that the cell pellets are fully resuspended; firm pellets require longer vortexing. Repeat this procedure for the other tubes at intervals of 30 s.
4. After 3 min, transfer the first ethanol extract to the −40 °C cryostat to cool down. Repeat subsequently with intervals of 30 s, until all cell pellets have been extracted.

3.1.4 Further Sample Processing

In the protocol below, it is assumed that a Labconco RapidVap is used for the sample drying.

1. Turn on the cold trap of the RapidVap. Make sure the cold trap is empty. It will take 10–20 min to be ready.
2. Evaporate the ethanol/water mixture until the samples are dry. Set the speed of the RapidVap to 90%, and apply full vacuum.
3. 5 min after the start, switch on the heating and set to 30 °C.
4. 25 min after the start, decrease the vacuum to 5 mbar.
5. Stop the RapidVap 110 min after the start and check if the samples are completely dry. If not, continue until dry.
6. Resuspend the dried sediment in 500μL MilliQ water.
7. Mix thoroughly by vortexing and transfer to Eppendorf tubes.
8. Centrifuge at 15,000 × *g* for 5 min at 1 °C. (If the supernatant is still turbid, transfer supernatant to clean Eppendorf tubes and centrifuge again.)
9. Transfer the supernatants to (labeled) 0.2-μm Durapore PVDF centrifuge filters.
10. Filter by centrifuging again at 15,000 × *g* for 5 min at 1 °C.
11. Transfer supernatant to screw-cap sample vials and store at −80 °C until analysis.

3.2 Rapid Sampling for Endometabolome Analysis: Cold Filtration Method

For quantification of intracellular metabolites which are present in the cells in very low amounts compared to their presence in the cultivation medium, the washing efficiency of the cold centrifugation method may not be sufficient. Therefore, a method was developed whereby cold methanol quenching is combined with a cold filtration step for virtually complete removal of the exometabolome [12]. *See* Fig. 1 right panel for a schematic overview of the method. This procedure is especially useful to quantify intracellular amounts of substrates and secreted (by)products. In the following protocol, it is assumed that 60% aqueous methanol is a suitable quenching

and washing liquid (*see* **Note 6**), that boiling ethanol/water is a suitable extraction method (*see* **Note 5**), and that samples with a volume of 10 mL are required. A different quenching liquid and an adjusted sample volume can be used provided that the temperature after sampling is kept at −20 °C or lower, to prevent enzymatic conversion of metabolites. As timing during this method is critical, the sampling is best carried out with two experimenters. Although the description of sampling and extraction is divided over two sections, both should be carried out quickly and smoothly in one go. The entire procedure, from sampling until submerging the filter containing the cells into the 75% ethanol, should be carried out fast enough to prevent the sample from warming up.

3.2.1 Preparation

For n samples, prepare the following the day before the sampling is carried out:

1. $3 \times n$ tubes with 50 mL of 60% methanol. Cap and cool down to −40 °C.
2. n tubes with 30 mL of 75% ethanol. Cap and heat them up in a 70 °C water bath before the sampling starts. (70 °C is just below the boiling point of this mixture.)

The next steps are best performed on the same day the sampling is performed:

1. Place the vacuum filtration unit on the balance (*see* Fig. 1, right panel). Connect the tubing to the vacuum pump without strain, such that it does not affect the weight of the filtration unit during sampling.
2. Calibrate the pipette required for ^{13}C extract additions (typically 100μL).
3. If IDMS is applied for metabolite quantification (*see* **Note 2**): Let the frozen ^{13}C-labeled extract thaw in the fridge. Make sure that you use the same uniform solution for all samples and standards. Keep the vial containing the ^{13}C extract closed and cold on ice.

3.2.2 Sampling

1. Place a filter on the filter support disc and clamp the filtration beaker.
2. Open a tube with 75% ethanol at 70 °C (required for extraction in a few minutes) and keep it in the 70 °C water bath.
3. Get three tubes with 50 mL of 60% methanol at −40 °C from the freezer/cryostat. Leave two of them next to the sampling setup ready to grab and pour out one in the filtration beaker, for washing the cell cake.
4. Tare the balance.

5. Switch on the peristaltic pump and flush the dead volume of the sampling tubing into a waste tube. Without switching off the pump, direct the flow/spray into the cold 60% methanol in the filtration beaker. The spray must directly contact the cold 60% methanol, so avoid hitting the wall of the filtration beaker. Switch off the pump after approximately 10 g (= 10 mL) of broth has been sampled.
6. Read the exact sample weight from the balance. (The second experimenter has time to write down the weight.)
7. Start the vacuum pump. Open the second 60% methanol tube while the broth/methanol suspension is filtered and pour it out into the beaker only after the filter cake falls dry. Repeat with the third 60% methanol tube and turn off the vacuum pump after the filter cake falls dry.

3.2.3 Extraction of the Cell Cakes

1. Remove the filtration beaker, lift up the filter with cell cake using tweezers, pipette 100μL of ^{13}C extract (0 °C) on top of the washed cell cake and immediately submerge the cell cake in the 75% ethanol tube at 70 °C.
2. Cap the tube and vigorously shake it by hand for 5 s (glass fiber filters will disintegrate at this point) and then place in a 95 °C water bath for 3 min (open the cap slightly to prevent pressurization).
3. Remove the tube from the water bath and cool it on ice. Recap the tube.
4. If desired, the sample can now be stored at −80 °C until further processing. If not, continue with Subheading 3.2.4, **step 1**.
5. Clean the filtration setup for the next sample.

3.2.4 Further Sample Processing

In the protocol below, it is assumed that a Labconco RapidVap is used for the sample drying.

1. Centrifuge the extracted samples for 8 min at 4 °C and 4400 × *g*.
2. Filter the supernatant using a 0.2-μm filter to remove glass fibers from the solution.
3. Evaporate the thus obtained extract to dryness using the RapidVap. Alternatively, if problems occur with resuspension of the dry residue, the extract can be concentrated instead of complete evaporation to dryness, e.g., to a final volume of 300–500μL. The drying/concentration step requires about 2 h (depending on the number of tubes processed at the same time). *See* Subheading 3.1.4 for the steps preparing the RapidVap for use. Start at a slow speed (30%) and increase as more and more water and ethanol evaporates. Set the heat to

30 °C. Do not apply full vacuum at once, but start at 200 mbar and decrease the pressure in steps of 20 mbar every 20 s until full vacuum.

4. Resuspend the residue in 500μL MilliQ water (or fill up to 500μL, if the extract is not evaporated to dryness).
5. Mix thoroughly by vortexing and transfer to Eppendorf tubes.
6. Centrifuge at 15,000 × *g* for 5 min at 1 °C. (If supernatant is still turbid, transfer supernatant to clean Eppendorf tubes and centrifuge again.)
7. Transfer the supernatants to (labeled) 0.2μm Durapore PVDF centrifuge filters.
8. Filter by centrifuging again at 15,000 × *g* for 5 min at 1 °C.
9. Transfer supernatant to screw-cap sample vials and store at −80 °C until analysis.

3.3 Rapid Sampling for Exometabolome Analysis

With this procedure, samples from a culture of microorganisms are quickly cooled down to a temperature close to 0 °C. The purpose is to minimize metabolic activity as much as possible while avoiding freezing the sample, as this may lead to cell damage. The cooling of the sample is accomplished by direct contact with pre-cooled steel beads which are placed in a syringe. Directly thereafter the sample is pressed through a filter to obtain a supernatant sample. The amount of beads needed to cool down the sample to a temperature slightly above 0 °C can be calculated from the heat capacities of stainless steel and water, the required sample volume, the initial sample temperature, and the initial temperature of the stainless steel beads, *see* ref. 49. Note that if the cells are susceptible to cold shock (i.e., sudden cooling will result in release of metabolites from the cells), the cooling step should be omitted. The protocol below is designed for the withdrawal of 2 mL of sample with an initial temperature of 30 °C.

3.3.1 Preparation

1. Fill the required number of syringes with 25 g of stainless steel beads each. Close the syringes with their plungers and the syringe outlets with parafilm and put them overnight in a freezer at −20 °C.

3.3.2 Sampling

1. Take the required number of syringes filled with cold beads from the freezer, remove the parafilm, and connect the filters to the syringes. Keep them in a Styrofoam box filled with cooling elements of −20 °C until sampling, to prevent them from warming up.
2. Sample 2 mL of broth from the bioreactor into a syringe and filter immediately, while collecting the supernatant in a sample vial.

3. Store the sample at −80 °C until analysis. Alternatively, if compounds should be quantified which may be susceptible to enzymatic conversion, it is advisable to destroy possible enzymes present by boiling ethanol extraction as described in Subheading 3.4.3.

3.4 Differential Method

This method is to be preferred if cold methanol quenching results in significant leakage of metabolites from the cells as might be the case for prokaryotic organisms [13–15]. To be sufficiently accurate, it is essential to combine this method with IDMS for metabolite quantification (*see* **Notes 2** and **7**).

With the differential method, each measurement requires two samples: a total broth sample and a filtrate sample. However, in particular cases, depending on the experimental design, the extracellular metabolite levels may be assumed to be in pseudo steady state, which means that they do not change significantly during the time a series of samples is taken. Then only a few samples are required to quantify the extracellular metabolite levels (*see* ref. 30). For the protocol below, it is assumed that 1 mL of sample is required for measuring the metabolites in total broth and 2 mL is required for measurement in the culture filtrate. Clearly, these amounts may differ from case to case and depend on the sensitivity of the analysis method applied. Thereby it must be taken into account that application of this protocol results in a six times dilution of the sample, while the conventional boiling ethanol/water protocol for cell extraction results in a two times concentration. For further comments on the applicability of the differential method, *see* **Note 7**.

3.4.1 Preparation

Most convenient is to carry out the following preparatory steps the day before the sampling is performed:

1. For *n* samples, prepare:
   - *n* tubes containing 5 ml of 60% v/v MeOH for sampling. Number and weigh them. Store at −40 °C.

   Only if rapid cooling of the sample is required and the microorganisms are not susceptible to cold shock (*see* refs. 13, 15):
   - *n* syringes filled with the proper amount of stainless steel beads (*see* protocol for exometabolome sampling). Close the syringes with their plungers, cover the syringe outlets with a layer of parafilm (to prevent formation of ice) and leave them overnight in a freezer at −20 °C.
   - *n* tubes containing 5 ml of 75% v/v EtOH for the extraction step. Store in the fridge.
2. Turn on the cryostat and set the temperature to −40 °C.

3. Connect the rapid sampling setup to the bioreactor from which the total broth samples should be withdrawn. Make sure that the bioreactor contains a second sampling port for withdrawal of broth to obtain the filtrate samples.

The next steps are best performed on the same day the sampling is performed:

1. Let the frozen ^{13}C-labeled extract thaw in the fridge. Make sure that you use the same uniform solution for all samples and standards (*see* **Note 2**). Keep the vials containing the ^{13}C extract closed and cold on ice.
2. Place the tubes containing 60% (v/v) methanol, required for sampling in the cryostat at −40 °C.
3. Adjust the timer of the rapid sampling system such that the weight of the sample taken equals the desired amount, in this case: 1.0 ± 0.05 g.
4. Calibrate the pipette required for ^{13}C extract additions (typically 100μL).
5. Switch on a suitable water bath and let it reach a temperature of 95 °C before sampling is started.
6. Only if rapid cooling of the sample is required: Take the required number of syringes filled with cold beads from the freezer, remove the parafilm and connect the filters to the syringes. Keep them in a Styrofoam box filled with cooling elements of −20 °C until sampling, to prevent them from heating up.

3.4.2 Sampling

1. Withdraw 1.0 mL of broth into a sampling tube (containing 5 mL of 60% methanol at −40 °C) using the rapid sampling device, mix directly after sampling by vortexing, and place the tube back in the cryostat.
2. Withdraw approximately 2 mL of broth from the bioreactor into a syringe.
3. Filter the sample immediately thereafter by pressing the sample through the filter and collect the supernatant in a sample vial. Pipette 1 mL of filtrate in a sampling tube (containing 5 mL of 60% methanol at −40 °C) mix thoroughly by vortexing and place the tube back in the cryostat.
4. Repeat **steps 1–3** for the number of measurements required.
5. Weigh each tube for exact sample amount determination (by subtracting the weight of the tube containing the 5 mL of 60% methanol determined the day before) and put back in the cryostat. Make sure the cryostat fluid (e.g., ethylene glycol) is effectively wiped from the walls of the tube as it can affect the weighing and lead to overestimation to sample weight

(especially with small sample volumes), *see* **Note 3**. The weighing procedure should be expeditious to prevent warming up of the sample.

3.4.3 Extraction of the Quenched Total Broth and Filtrate Samples

In this protocol not only the total broth sample but also the filtrate sample is extracted in hot ethanol, to denaturate all possible enzymes present (*see* **Note 5**). Even the presence of minimal amounts of enzymes would lead to distortion of metabolite profiles later on in the sample processing, which must be avoided.

1. Transfer from each quenched broth sample 500μL into an empty tube and keep them in the cryostat at −40 °C until extraction. Be sure to completely mix the quenched samples by vortexing before the transfer.
2. Repeat this procedure for the quenched filtrate samples.
3. Add the U-^{13}C internal standard mix (typically 100μL).
4. Apply the same procedure as described for extraction of the cell pellets (*see* Subheading 3.1.3).

3.4.4 Further Processing of the Total Broth and Filtrate Samples

Apply the same procedure for sample drying and cleanup as described for the cell pellets (*see* Subheading 3.1.4).

3.4.5 Determination of the Intracellular Metabolite Levels for the Differential Method

After quantification of the metabolites in the total broth and filtrate samples, the intracellular amounts can be calculated by subtraction. Proper quantification of the real amounts of sample taken, which was performed by weighing in case of the total broth samples and by accurate pipetting (in addition, weighing can be used here) will increase the accuracy of the final result. The most convenient way of expressing the metabolite levels, both in total broth and in the filtrate, is per amount of biomass present in the bioreactor, e.g., in μmol per gram of biomass dry weight. Subtraction of metabolite levels in the filtrate from the total broth levels then directly yields intracellular levels (*see* **Note 7**).

3.5 Principles of Metabolite Quantification Using Isotope Dilution Mass Spectrometry

A detailed description of how to apply isotope dilution mass spectrometry will not be given here. Different methods for the analysis of different groups of metabolites have been published previously [47, 48]. The principle of the method is that metabolites are quantified by mass spectrometry, whereby for each individual metabolite a chemically identical, fully ^{13}C-labeled analog is added as internal standard. Each metabolite is then quantified relative to the amount of its fully ^{13}C-labeled analog present. This procedure effectively corrects for non-idealities in the subsequent MS-based quantification, such as sample matrix effects, nonlinearity resulting from competition in the ESI interface (in case of LC-MS analysis), incomplete derivatization (in case of GC-MS analysis), machine drift, etc.

To allow quantification, the ^{13}C-labeled internal standard mix is therefore added to the samples, as well as to a series of dilutions of a conventional standard mix. If the ^{13}C-labeled internal standard mix is added to the samples before the extraction procedure, partial degradation of metabolites, as well as partial losses of sample, e.g., by transferring them to different tubes, are also corrected for. Unfortunately, for most metabolites fully ^{13}C-labeled analogs are not commercially available. The only way to obtain ^{13}C-labeled analogs for all metabolites to be measured is therefore to carry out a cultivation on a medium containing a fully ^{13}C-labeled carbon source, e.g., 100% U-^{13}C-labeled glucose [47]. Extraction of the cells will then yield a U-^{13}C-labeled metabolite mixture which can be used as internal standard.

4 Notes

1. *The necessity of fast sampling and quenching.*

   To obtain a proper quantitative snapshot of the microbial metabolome, fast sampling is essential. It should be realized that as soon as a sample is withdrawn from a culture, the conditions to which the cells are exposed will change, e.g., with respect to temperature, substrate and oxygen availability, carbon dioxide pressure, and pH. For example, in a sample taken from a high-density aerobic batch cultivation, the available dissolved oxygen might be depleted within 1 or 2 s. The same holds for the substrate concentration in a sample withdrawn from a substrate-limited chemostat culture. Changes in the environment of the cells will result in changes in metabolic rates. Because the vast majority of metabolites have turnover times of seconds or less, this will result in changes in the metabolome. Therefore, to prevent these changes, the time between withdrawal of the sample and quenching of all metabolic activity should preferably be less than a second.

2. *Use of ^{13}C extract as internal standard.*

   For a proper quantification of metabolites with IDMS, the amount of U-^{13}C extract added should be such that, after the addition, the concentrations of the U-^{13}C-labeled analogs in the sample are in the same range as the metabolites which have to be quantified. This should be taken into account in the preparation of the ^{13}C extract, i.e., in the final concentration of the extract. In some cases, it might be required to either dilute the ^{13}C extract or the samples to achieve this.

   It is important that the same ^{13}C extract, i.e., from the same batch, is used for all samples and standards of the same series. Furthermore, repeated freezing and thawing of the extract may result in partial degradation of metabolites,

whereby the extent of degradation is metabolite-specific. Ideally, the addition of ^{13}C extract to the samples and standards should be performed on the same day, from a single pool of extract. If this is not practical (e.g., when samples need to be taken on separate days during cultivation, to observe long-term trends in metabolite levels), the ^{13}C extract to be used should be distributed over several vials and frozen together. A single aliquot can then be thawed on the day it is needed. Note that, due to the concentration step, cell extracts are often rather viscous solutions, so be careful when the ^{13}C extract is added to the samples by pipetting. It should be stressed here that the precision of the end results depends on the accuracy of the ^{13}C extract addition, because they are calculated based on that value. If the precision of the pipettes used is found to be insufficient, positive displacement pipettes may provide a better alternative, because they are more suitable for working with viscous solutions than air/piston pipettes.

3. *Determination of the exact sample amount by weighing.*

   No matter which sampling device is used to rapidly withdraw samples from a bioreactor, the exact amount withdrawn will vary between certain limits. For an accurate quantification of metabolites, it is therefore essential to determine the exact amounts of sample withdrawn by weighing the tubes containing the cold aqueous methanol solution before and after sampling. Because cold methanol is very hygroscopic, water vapor will quickly condense within the tube, affecting the weight of the tubes. Therefore, all tubes containing cold aqueous methanol should be kept closed and should only be opened shortly for sampling.

4. *Occurrence of leakage of metabolites into the quenching solution.*

   In the protocols above, it is assumed that 60% methanol is a suitable quenching liquid. However, some authors have reported that this quenching solution may give rise to metabolite leakage, even for eukaryotic cells [15, 31]. The suitability of a quenching liquid should therefore beforehand be validated, preferably in a quantitative way [15, 31]. Shortly, this involves comparing the intracellular amounts measured after using the differential method to the intracellular amounts measured after using either the centrifugation or the filtration method. If in addition to this also metabolite quantification in the quenching and washing solutions is carried out, this allows to calculate the full mass balances and to quantify the extent of leakage for each metabolite as has been described extensively in Canelas et al. [16], *see* Fig. 2. If leakage is detected, this mass balance approach can be used to test systematically the effect of changes in the properties of the quenching solution (temperature, concentration of solvent, ionic strength, etc.) and/or the cell

separation method (centrifugation or filtration) and optimize the whole-quenching procedure to prevent the occurrence of leakage.

5. *Metabolite extraction.*

   In the protocols described above, boiling in hot aqueous ethanol has been applied to extract the metabolites from the cell samples. This method has several advantages compared to other published extraction procedures. Because the extraction is carried out at a high temperature, all enzymes present are denaturated, preventing further enzymatic (inter)conversions of metabolites in the cell extract. Furthermore the extractant (ethanol/water) is nontoxic and easily removed by vacuum evaporation.

   It has been shown that for methods, e.g., freeze thawing in methanol (FTM), for which complete inactivation of all enzymatic activity is not guaranteed [44] significant changes in metabolite concentrations will occur. Nevertheless FTM extraction has been applied in several published studies, *see* ref. 50 and references therein.

6. *Quenching in cold aqueous methanol.*

   At present, the cold methanol quenching method is widely considered as the most suitable procedure which allows the removal of the compounds which are present in the cultivation medium. To be applicable for a certain organism, the cells should remain intact during the quenching procedure and metabolite loss into the quenching solution should be negligible, otherwise no meaningful measurements will be obtained. Removal of extracellular compounds is important in different aspects. First of all, thanks to the increased sensitivity of the, mainly MS-based, analytical instruments, it has become evident that many metabolic intermediates are also present in the cultivation medium. Although the concentrations in the medium are in most cases at least two orders of magnitude lower than the intracellular concentrations, the medium volume is so much larger than the total cell volume (roughly two orders of magnitude in most laboratory cultivations) that the total amounts of these metabolites which are dissolved in the medium can still be very significant. This implies that if the cells are not separated from the surrounding medium prior to metabolite extraction, the resulting metabolome measurements are not representative for the intracellular levels. Another reason to remove extracellular compounds before extraction is that certain medium constituents, e.g., sulfate, phosphate, and chloride, may interfere with the analysis method applied for metabolite quantification. For example, when LC-ESI-MS/MS is applied, whereby anion exchange chromatography is used for the LC separation, these

compounds may elute together with the metabolites of interest, thereby not only affecting their retention times but also decreasing the sensitivity of the MS due to competition in the ESI interface (*see* ref. 44). Clearly, the possible intervention of sample constituents with the analysis method to be applied should be a point of attention.

7. *Applicability of the differential method*.

To be able to apply the differential method for quantification of the endometabolome, whereby the intracellular metabolite levels are determined by subtraction of metabolites quantified in total broth and in culture filtrate, some boundary conditions have to be fulfilled. First of all, the intracellular level of a metabolite can only be quantified with reasonable accuracy if the amount present inside the cells is significant compared to the amount present outside, i.e., in the culture medium (both expressed in amount per amount of biomass present). It should be clear that if for example more than 90% of a metabolite is present outside the cells, quantification with the differential method will not produce reliable results. Furthermore, a proper determination of the measurement errors is required to be able to calculate the error in the end result. To minimize these errors, it is strongly advised to apply IDMS for the quantification of the metabolite levels in total broth and supernatant.

Another issue connected with the application of the differential method is that all components present in the cultivation medium are also present in the samples. If some of these interfere with the analysis (*see* **Note 6**), the samples should be diluted before analysis. This was the reason that we applied a dilution step in the described protocol for the differential method. Clearly, thereby the applied analysis procedure should be sensitive enough to quantify the metabolites in the diluted samples. It should be stressed that this issue is specific for the applied analysis method as well as for the composition of the cultivation medium and possible by-product formation by the cells and should be verified beforehand.

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