Chapter 8

ID-MS-Based Quantitative Analysis of Metabolites in *Pichia pastoris*: A Step-by-Step Protocol

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Abstract

Quantitative metabolomics is based on a set of experimental approaches to accurately quantify intracellular metabolite concentrations. This allows us to characterize the response of a metabolic network (i.e., the metabolic phenotype) to an environmental or genetic perturbation. Here, we describe a four-step protocol adapted to the methylotrophic yeast *Komagataella phaffii:* (1) separation of the cells from the fermentation broth by cold filtration and addition of ^{13}C-labeled cell extract, (2) a metabolic quenching step based on aqueous cold methanol, (3) a metabolite extraction method based on boiling ethanol, and (4) quantification by isotope dilution mass spectrometry (LC-IDMS/MS and/or GC-IDMS). This method allows us to quantify most metabolites of central carbon metabolism, including glycolytic, tricarboxylic acid cycle, and pentose phosphate pathway intermediates, as well as cofactors and free amino acids. This method has been validated for *K. phaffii* grown on glucose, as well as on a mixture of carbon substrates such as methanol in combination with glucose or glycerol.

Key words *Pichia pastoris*, *Komagataella phaffii*, Metabolomics, Microbial metabolism, Intracellular metabolites, ^{13}C-labeled internal standard, Quenching, Extraction, Isotope dilution mass spectrometry

1 Introduction

In past years, metabolomics has developed methods for the quantitative and broad analysis of microbial metabolism, mainly due to improvements in MS-based analytical procedures [1–4]. Together with advances in the analysis of intracellular fluxes via ^{13}C isotopic labeling [5], a quantitative analysis of metabolic pathways is well within reach.

To study the function of metabolic reaction networks, it is crucial to obtain a representative and accurate snapshot of the quantitative state of metabolic pathways. Most metabolites, especially those of the central carbon metabolism, have turnover times in the order of seconds or less, which highlights the need for a rapid

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sampling protocol to properly capture a representative cultivation state. Next to rapid sampling and inactivation, sampling protocols must ensure that there are no losses of metabolites during the sampling (leakage-free quenching) [6, 7] and the subsequent extraction procedure. Additionally, the intra- and extracellular space should be well separated, e.g., if substantial amounts of metabolites are present in the extracellular medium, those need to be efficiently removed. Therefore, a sample treatment procedure allowing separation of the intracellular and extracellular metabolite pools, as well as a degradation-free extraction method, is required [8].

Although many efforts have been directed toward the development of a universal method, no consensus solution has been found because of the vast diversity in cell properties [7, 9, 10].

Rapid sampling, quenching, separation, and extraction protocols for the intracellular metabolite quantification in *Komagataella phaffii* (also known as *Pichia pastoris*) have been presented and successfully applied over the past years, allowing accurate, reliable, and reproducible metabolite determinations [11, 12].

Here we provide detailed protocols for the quantitative analysis of the *K. phaffii* endo- and exometabolome, based on the work of Carnicer et al. [11]. They cultivated the cells under well-controlled conditions (growth rate, pH, oxygen supply) in steady-state chemostats and optimized/validated rapid sampling, cold methanol-based quenching, and hot ethanol-based extraction procedures for *K. phaffii*. Metabolite quantification was carried out by isotope dilution mass spectrometry (LC-IDMS/MS and GC-IDMS) [13]. Validation of the procedure was performed through quantification of metabolites in all different sample fractions (whole broth, quenched and washed cells, quenching and washing solutions, and culture filtrate) combined with a mass balance approach. The optimized protocols can be applied for quantitative analysis of intra- and extracellular metabolites in *K. phaffii* under dynamic conditions as well as ^{13}C-labeling experiments, including instationary metabolic flux analysis [14, 15].

Overall, the detailed procedure includes (1) fast sampling from a yeast culture and quenching using cold methanol, (2) separation and washing of the cells by cold filtration and addition of ^{13}C-labeled cell extract as an internal standard [16], (3) extraction of intracellular metabolites using boiling ethanol, and (4) a metabolite quantification and data analysis step. Specifically, the sampling process described here assumes that the cells are grown in well-controlled bioreactors (growth rate, pH, pO_2, etc.) and with online monitoring. Moreover, the bioreactors should allow fast sample collection, which can be best achieved by a sampling port in direct proximity to the culture broth, with a short tubing distance between the sample port and quenching solution, and overpressure inside the bioreactor. Notably, in this protocol cells are separated

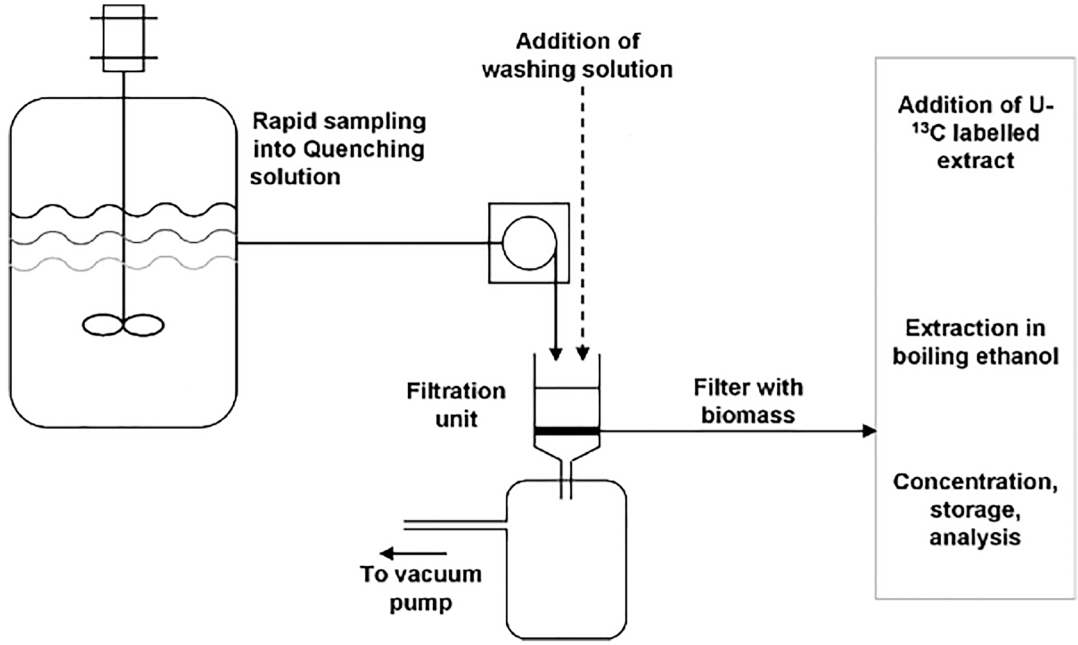

Fig. 1 A schematic overview of the cold filtration based sampling and processing procedure (taken from [17]). Rapid sampling is performed using the device of Lange et al. [18] using under pressure. Remark: The double valve to apply under pressure on the sample tube should be included

from the fermentation broth and washed by a cold filtration-based procedure, in contrast to other protocols based on cold centrifugation [13]. Figure 1 shows a schematic overview of the sampling procedure based on cold filtration performed in *K. phaffii*. Possible alternative sampling set-ups to facilitate the fast sampling and cell quenching should be evaluated before starting the experiment.

A comprehensive protocol for ID-MS-based quantitative metabolomics has been previously published by Wahl and co-workers [13], using *S. cerevisiae* as a reference case.

2 Materials

2.1 *Cultivation of* K. phaffii

The cultivation procedure and sampling scheme (number of samples, frequency, volume, cell amount) for metabolome profiling will vary depending on the purpose and needs of the study. The desired setup for the following metabolomics protocol is a fully instrumented bench-top bioreactor, to which a fast-sampling workflow procedure can be easily implemented (*see* **Note 1**).

2.2 Materials Needed for Sample Preparation

1. Quenching solution: 60% (v/v) methanol.
2. Extraction solution: 75% (v/v) ethanol.

3. ^{13}C internal standard solution, for example, ^{13}C-labeled cell extract [3, 16]. The internal ^{13}C must contain all relevant metabolites as U-^{13}C-labeled isotopes.
4. Membrane disk filters 47 mm in diameter, 0.45μm pore size (Pall Corporation, USA).
5. 0.2μm Durapore PVDF centrifuge filters.
6. 2-mL Eppendorf tubes.
7. Polypropylene test tubes (14 mL, 17 mm diameter).
8. 50-mL Falcon tubes.

2.3 Equipment for Sample Preparation

1. Bench-top bioreactor equipment, preferably equipped with CO_2 and O_2 off-gas analyzers.
2. Spectrophotometer equipped with a visible (400–700 nm) light source.
3. HPLC equipment, ion exchange column (e.g., Aminex HPX-87H, Bio-Rad Laboratories, Inc.), and HPLC vials (HPLC is used for extracellular metabolite analyses).
4. Rapid sampling setup for the bioreactor, e.g., as described in Fig. 1 [18]. The use of this device will allow controlling the sample volume taken from the bioreactor as well as reducing the residence time of the cells in the tube before being quenched.
5. Cryostat at −27 °C, the optimal temperature for quenching *K. phaffii* cells [11].
6. RapidVap (Labconco, USA) to evaporate/concentrate the samples. The amount of the metabolome samples that are going to be evaporated will be 30 mL. Therefore, the RapidVap should be adjusted to be able to cope with these volumes.
7. Refrigerated bench-top centrifuge for 2-, 15-, and 50-mL tubes.
8. 2× water baths. Operating conditions: 75 °C and 95 °C.
9. Vacuum filtration setup.

2.4 Materials and Equipment for LC-MS/MS Analyses

The LC-MS/MS and GC-MS analytical platforms used in this method have been recently described in Wahl et al. [13].

2.4.1 LC-MS/MS-Based Analysis for Glycolytic and Tricarboxylic Acid (TCA) Cycle Intermediates

1. HPLC: Alliance HT pump 2795 system (Waters, USA).
2. MS: Quattro LC™ triple quadrupole (Waters, UK).
3. 515 HPLC pump (Waters, USA).
4. Two-position ten-port stainless steel LabPro Selector valve (Rheodyne, USA).
5. Two-way LabPro Solvent select (Rheodyne, USA).

6. ASRS® 300 4 mm self-regenerating suppressor (Thermo Scientific, USA).
7. HPLC propylene screw top 300µl vials with cap and bonded pre-slit PTFE/silicon septa (Waters, USA).
8. Sodium hydroxide.
9. Methanol, HPLC grade.
10. LC column for glycolytic and TCA cycle metabolites: IonPac AS11 (Dionex, Sunnyvale, USA) 4 mm inner diameter column to 250 mm in length.
11. Guard column: An XTerra® MS C18 column with a 3 mm inner diameter and 20 mm length (Waters, Ireland).
12. Polypropylene sample vials (Waters).

2.4.2 LC-MS/MS-Based Analysis for Nucleotides and Coenzymes

1. HPLC: Alliance HT pump 2795 system (Waters, USA).
2. MS: Quattro Premier XE triple quadrupole mass spectrometer equipped with an electrospray ion source (Micromass MS Technologies—Waters, UK).
3. 515 HPLC pump (Waters, USA).
4. Ten-port Selector valve (Waters, USA).
5. LC column for analysis of nucleotides and coenzymes: AcQuity™ UPLC® BEH C18 (1.7µm, 100 mm × 2.1 mm i.d., Waters, Ireland).
6. Guard column: VanGuard™™ pre-column, AcQuity UPLC® BEH C18 (1.7µm, 5 mm × 2.1 mm, Waters, Ireland).
7. Polypropylene sample vials (Waters).

2.4.3 The GC-MS/MS-Based Analysis for Amino Acids Includes

1. GC: Agilent GC system 7890 A (Agilent Technologies, CA, USA).
2. MS: Agilent 5975C quadrupole MS equipped with an electron ionization (EI) ion source (Agilent Technologies, CA, USA). MassHunter software is used to control the MS and spectral processing.
3. Agilent automatic liquid sampler 7683B (Agilent Technologies, CA, USA).
4. Programmed temperature vaporizing (PTV) injector (Gerstel, Mühlheim, Germany), equipped with a glass liner containing glass wool.
5. GC column: ZB-50 column (30 m × 0.25 mm × 0.25µm, Phenomenex, CA, USA).
6. O-methoxyamine hydrochloride (MOX) stored in desiccators.
7. Anhydrous pyridine.

8. N-Methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) for amino acid derivatization.
9. Glass sample vials (Agilent Technologies).

3 Methods

3.1 K. phaffii Cultivation

Grow the desired *K. phaffii* strain in the required conditions (*see* **Notes 1** and **2**).

3.2 Before Sampling

1. For each intracellular metabolome sample prepare:
   (a) *1 sampling tube*: 5 mL of quenching solution in 14-mL polypropylene tubes. Number and weigh them. Store at −40 °C.
   (b) *1 washing tube*: 10 mL of quenching solution in 14-mL polystyrene tubes. Store at −40 °C.
   (c) *1 extraction tube*: 30 mL of extraction solution in 50-mL Falcon tubes. Store at 4 °C. (*see* **Note 3**)
2. Turn on the cryostat at −27 °C, preferably the day before the sampling.
3. Connect the rapid sampling setup to the bioreactor and calibrate the valves for sampling the required broth volume (*see* **Note 4**).

3.3 Rapid Sampling

1. Take the ^{13}C-labeled extract from −80 °C and let it thaw at 4 °C (*see* **Note 5**).
2. Place the sampling tube and washing tube in the cryostat.
3. Set each of the two water baths at 75 °C and 95 °C, respectively.
4. Place the extraction tube in the 75 °C water bath and wait 3–5 min to warm up the solution. Ethanol solution does not boil at that temperature.
5. Sample 600μL of broth, preferably using a rapid sampling setup, into a sampling vial (containing 5 mL of 60% methanol at −27 °C).
6. Weigh the sampling tube for calculation of the exact sample weight (*see* **Note 6**).
7. Filter the cell suspensions through 0.45μm pore size membrane disk filters (47 mm diameter) by using a vacuum pump.
8. Wash the filters to remove as much extracellular metabolites as possible by pouring 10 mL of quenching solution (washing tube) on the filter cake as soon as the cake starts to fall dry.

9. Pipette 120μL of ^{13}C-labeled extract on top of the dry filter cake (*see* **Note 6**).
10. Once the filter cake looks dry, stop the vacuum pump and transfer the filter cake into the extraction tube at 75 °C.
11. Place the extraction tube containing the filter in the 95 °C water bath for 3 min.
12. Remove the extraction tube from the water bath and place it back in the cryostat (*see* **Note 7**).
13. Perform dry cell weight (DCW) analysis to know the extract biomass concentration in the bioreactor.
14. After sampling: Metabolite extraction.
15. Turn on the RapidVap and wait until it is ready.
16. Remove the filter disk from the extraction solution.
17. Place the extraction tubes in the RapidVap and follow the standard protocol for drying.
18. Meanwhile, cool down the Eppendorf centrifuge at 1 °C.
19. When the drying is completed, take the samples from the RapidVap and resuspend the sediments with 600μL of ultrapure water.
20. Transfer the resuspended solutions to Eppendorf tubes and centrifuge them at 12,000 × *g* for 5 min at 1 °C.
21. Transfer the supernatants to 0.2μm Durapore PVDF centrifuge filters.
22. Filter by centrifugation again at 12,000 × *g* for 5 min at 1 °C.
23. Transfer the filtrate into screw-cap sample vials.
24. Store the samples at −80 °C until analysis.

3.4 Preparation of Standards: Calibration Curves

The calibration curves for analysis of metabolites are constructed based on the ratio between the ^{12}C-metabolite/U-^{13}C-metabolite areas. The U-^{13}C-metabolites are used as internal standards to overcome potential metabolite losses or degradation during sample extraction, inefficient derivatization, matrix effects during analytical measurements, etc. [16]. Therefore, it is important to use the same ^{13}C-labeled cell extract used in the samples when preparing the standards.

The metabolite concentrations in the samples can vary depending on the metabolite being an intermediate or end product, the culture conditions, the cell density, the sample volume, etc. Therefore, the standard calibration ranges must be adjusted accordingly. Normally, 50–100μM is sufficient as the highest metabolite concentration in the calibration curves for the metabolites present in glycolysis (e.g., glucose 6-P, fructose bisphosphate, dihydroxyacetone, etc.), using this metabolome protocol for *K. phaffii* grown in

glucose-limited chemostat cultures on glucose at a steady-state biomass concentration of about 4 g_{DCW}/L.

In addition, to facilitate the calculations it is preferred that the ratio between the total volume of each standard (e.g., 100μL) and the volume of ^{13}C-labeled extract added (e.g., 20μL) to the standards is equal to the ratio between the total volume of each sample after resuspension (e.g., 600μL) and the volume of ^{13}C-labeled extract added to the samples (e.g., 120μL). If this is not the case, a correction factor (CF) needs to be applied (*see* Subheading 3.7).

3.5 Metabolite Quantification with Mass Spectrometry

Quantitative analyses of metabolites are conducted using isotope dilution mass spectrometry-based analytical platforms (LC-IDMS/MS and GC-IDMS).

LC-IDMS/MS analysis of glycolytic and TCA metabolites is performed based on a modified method reported by van Dam et al. [1]. Briefly, a Waters Alliance HPLC 2795 system (Waters, USA), coupled to an ASRS® 300 4 mm self-regenerating suppressor (Thermo Scientific, USA) and a Waters Quattro LC triple quadrupole mass spectrometer (Waters, UK), is used. Metabolites are detected in negative ion mode with multiple reaction monitoring (MRM). Electrospray ionization is used as an interface between LC and MS. The capillary voltage is set at 2700 V; nitrogen gas is used as nebulizer gas at a flow rate of 88 L/h and as desolvation gas at a flow rate of 555 L/h with the temperature set at 250 °C. The ion block temperature is set at 90 °C. MRM transitions and corresponding instrument settings yielding the highest signal-to-noise ratio (S/N) are separately found for each individual metabolite by direct infusion of standard compounds into the MS.

GC-MS analysis of the pentose phosphate pathway metabolites was based on the method reported by [13, 19]. Briefly, a sample solution containing metabolites is lyophilized overnight, followed by derivatization reactions with 20 g/L MOX in anhydrous pyridine for 50 min at 70 °C. Then it is reacted for another 50 min at 70 °C with a mix of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) and trimethylchlorosilane (TMCS). The derivatized sample is centrifuged for 1 min at 10,000 × *g* and injected into the GC column using a PTV inlet program: initial temperature 70 °C, first ramp at 720 °C/min to 220 °C, hold for 5 min. A volume of 1μL is injected in splitless mode (for highly concentrated metabolites, in split mode 1/40 is used). The oven program is as follows: initial temperature 70 °C (hold for 1 min), then it is increased with a ramp of 10 °C/min to 320 °C with a final backflash of 5 column volumes at 320 °C. The MS setting is as follows: Transfer line 250 °C, source temperature 230 °C, and quadrupole temperature of 150 °C.

The MS/MS analyses of nucleotides and coenzymes are conducted based on methods reported by Seifar et al. [19, 21].

Briefly, a triple quadrupole mass spectrometer Waters Quattro Premier XE (Micromass MS Technologies-Waters) equipped with

an electrospray ion source in negative ion mode is used for the determination of metabolites with MRM. The capillary voltage is set at 2800 V; nitrogen gas is used as cone gas with a flow rate of 50 L/h as well as desolvation gas with a flow rate of 700 L/h at 360 °C. The ion block temperature is set at 120 °C. MRM transitions and corresponding instrument settings yielding the highest S/N ratio are separately found for each individual metabolite by direct infusion of standard compounds into the MS.

Amino acids are quantified using a method reported by de Jonge et al. [22], with GC-MS on an Agilent GC system 7890A (Agilent Technologies, CA, USA) coupled to the EI ion source of an Agilent 5975C quadrupole MS (Agilent Technologies, CA, USA). MassHunter software is used to control the MS and for spectral processing. Metabolites are detected and monitored in selected ion monitoring mode. The operating temperature of the quadrupole is set by default to 150 °C, and the ion source temperature is set to 230 °C with EI at 70 eV. The transfer line from GC to MS is kept at a fixed temperature of 250 °C.

Prior to GC-MS analysis, the amino acids are derivatized with MTBSTFA to volatile tert-butyldimethyl silane derivatives.

3.6 Liquid Chromatography

3.6.1 Anion Exchange Chromatography for Separation of Central Carbon Metabolites

Liquid chromatographic separation of glycolytic and TCA cycle metabolites is performed by anion-exchange chromatography using a method adapted from the method reported by van Dam et al. [1]. The analytical column is equipped with a pre-column to trap hydrophobic contaminants. The analytical column is maintained at a temperature of 25 °C, while the auto sampler temperature is set to 4 °C. The injection volume is 10μL with partial loop injection.

1. A sample volume of a minimum of 40μL is required.
2. Prepare four solvents: Mobile phase A: 5% (v/v) methanol, B: 0.6 mM NaOH, C: 60 mM NaOH, and D: 300 mM NaOH.
3. Set the flow rate of the LC to 1 mL/min with A: 95% and B: 5%.
4. Set the flow rate of the HPLC pump for the methanol inflow (80% v/v) to the ion suppressor to 0.7 mL/min.
5. Set the pump for solvent selector to 0.9 mL/min. The solvent selector switches between solvents of 80% (v/v) methanol and 5% (v/v) methanol.
6. Switch the ion suppressor on (auto suppression external water mode, the ion suppressor removes the sodium ions from the effluent of the analytical column). The final flow to the MS is reduced to ~100μL/min (T union flow splitter).

Table 1
Gradient profile used for anion exchange chromatography of central carbon metabolites

Time (min)	A%	B%	C%	D%
0	95	5	0	0
1.5	95	5	0	0
1.51	95	0	5	0
17	75	0	25	0
22	0	0	100	0
24	0	0	100	
24.2	0	0	0	100
30	0	0	0	100
31	95	5	0	0
38	95	5	0	0

7. The chromatography is started with the solvent selector in position A (the guard column is switched in front of the analytical column).
8. Inject 10μL of the sample.
9. The NaOH gradient profile is generated using the four solvent mixtures, and the gradient is programmed as described in Table 1.

In the first 1.5 min, the pre-column and separation column are connected in series. Then the pre-column is switched to the waste and washed continuously with an 80% (v/v) methanol solution. The effluent from the separation column is switched to the waste after 26 min, until the start of the next run. The ASRS® 300 4 mm self-regenerating suppressor is washed with a continuous flow of 80% (v/v) methanol until the end of the run.

3.6.2 Ion Pair Reversed-Phase Liquid Chromatography for Separation of Nucleotides and Coenzymes

The chromatographic separation of adenosine mono-, di-, and tri-phosphates, NAD, NADH, NADP, NADPH, CoA, acetyl CoA, succinyl CoA, and FAD in cell extracts is achieved on a reversed-phase column. A pre-column is employed to protect the analytical column from particles present in the sample solutions. The analytical column is maintained at 25 °C while the autosampler temperature is set at 4 °C. The gradient profile and mobile phase compositions are the same as described in Seifar et al. [20, 21] with minor modifications. The injection volume is set to 10μL with partial loop injection.

Table 2
Gradient profile used for IP-UHPLC of nucleotides and coenzymes

Time (min)	B%
0	2
2	2
8	100
8.1	2
15	2

IP-UHPLC Ion pair reversed-phase liquid chromatography

1. Transfer 50 mL of sample to an HPLC vial.
2. Add 1 mL of 50 mM ion-pair reagent DBAA to the sample vial.
3. Prepare two solvents: A: 5% (v/v) acetonitrile, 2 mM DBAA, and mobile phase B: 84% (v/v) acetonitrile, 2 mM DBAA.
4. Set the flow rate of the LC to 1 mL/min with A: 90% and B: 10%.
5. Set the HPLC pump for 50% (v/v) acetonitrile to a flow of 0.1 mL/min (located between the analytical column and the electrospray of the MS).
6. Using a T union flow splitter the final flow of the MS is reduced to 0.1 mL/min.
7. Inject 5 mL of sample into the LC column.
8. The gradient profile used is shown in Table 2.

The effluent from the analytical column, with a flow of 0.2 mL/min, is mixed with a flow of 0.1 mL/min of 50% (v/v) acetonitrile solution, which is provided by a Waters 515 HPLC pump before entering the electrospray ionization of the MS system. The make-up flow is applied to enhance the MS signal at the beginning of the chromatographic separation, when the organic solvent concentration is rather low. During the first two minutes and the last five minutes of each run, the effluent from the analytical column is diverted to waste to prevent contamination of the MS system by salts in the sample matrix and ion pairs in the mobile phases.

3.7 Gas Chromatography for Separation of Free Amino Acids

The gas chromatography (GC) separation of amino acids is based on the method reported by de Jonge et al. [22]. For GC-MS analysis, chemical derivatization is required, for instance, as described in the following steps. Additional experimental tips are provided in [13]:

1. Lyophilization: Transfer 100μL of sample into a glass sample vial, close with a screw cap with a Teflon septum (punch holes with a syringe needle), and freeze-dry overnight. Make sure that the samples do not start melting by, for instance, using a cold (−80 °C) block.
2. Dissolve MOX at 20 g/L in (anhydrous) pyridine; shake until MOX is completely dissolved (prepare fresh).
3. Add 50μL of 20 g/L MOX solution and incubate for 50 min at 70 °C.
4. Open a fresh MSTFA ampule and add 50μL of TMCS.
5. Add 80μL of MSTFA-TMCS and incubate for 50 min at 70 °C.
6. Transfer 100μL of the derivatized sample into a glass vial and centrifuge for 1 min at 10,000 × *g*.
7. Transfer 70μL to a GC vial with an inlet and close tightly with a screw cap.

For GC-MS analysis, the following operating conditions are used:

1. Injection and PTV inlet program: Samples are kept in an automatic liquid sampler. A 1-μL sample is injected into the separation column using a PTV injector equipped with a glass liner containing glass wool. The PTV is used with a split of 1:15 for samples with a high concentration of amino acids. The initial temperature of PTV is set to 100 °C and held for 0.2 min, then raised at a rate of 720 °C/s to 220 °C and maintained at that temperature for 5 min, and is finally raised at a rate of 720 °C/s to 300 °C and held for another 22 min.
2. Oven program: The initial oven temperature is set to 100 °C and held for 1 min and then raised at a rate of 10 °C/s to 300 °C. A backflush technique is used for cleaning up the column from slow-eluting compounds with high boiling temperatures. The backflush is conducted by reversing the carrier gas flow into the inlet and venting through the split line for 3.7 min. Then the oven temperature is reduced to 100 °C within 4 min to start the new run.
3. MS settings: The helium carrier gas flow is set to 1.5 mL/min. The transfer line to MS is kept at a fixed temperature of 250 °C.

3.8 Calculation of Intracellular Metabolite Concentrations

In metabolomics, the metabolite pool sizes are usually expressed as amounts per gram DCW (μmol/g_{DCW}). These units present are close to primary data; no assumptions on cell volume or compartmentation are required. A possibility to obtain the biomass dry weight (DCW) concentration is to use an OD measurement when the OD/g_{DCW} relation is known. However, the researcher needs to validate that this relation is valid for the culture conditions applied.

The amount per gram of biomass values ($c_{Mi/x}$) can be easily calculated as μmol/g_{DCW} knowing the cell density of the cultures c_X (expressed as g_{DCW}/L), the concentration of the metabolites in the sample (c_{Mi}) expressed as μmol/l, the broth sample volume (V_{sample}; 0.6 mL in this protocol), and the calculated sample volume taken from the bioreactor (V_t):

$$c_{Mi/x} = c_{Mi} \cdot \frac{V_{\text{sample}}}{V_t} \cdot \frac{1}{cx}$$

Finally, the metabolite pool sizes need to be corrected by the following CF:

$$\text{CF} = \frac{V\ ^{13}C_{\text{sample}}}{V_{\text{sample}}} \cdot \frac{V_{\text{standard}}}{V\ ^{13}C_{\text{standard}}}$$

where $V_{13Csample}$ is the volume of ^{13}C extract added to the cell pellet (μL) and $V_{standard}$ is the volume of ^{12}C standard (μL) with the respective addition of $V_{13Cstandard}$ of internal standard.

3.9 Preparation of ^{13}C Cell Extract

For the ^{13}C cell extract preparation, the content of ^{12}C must be minimized [3, 16]. Therefore, the cultivation is performed as fed-batch, starting with a low cell density (small inoculum) until a high cell density, fully grown on a ^{13}C carbon source, is reached. To balance the amount of intracellular metabolites, cells are harvested at different time points. Additionally, for the last harvest, a glucose pulse is applied to increase the amount of glycolytic intermediates in the extract. The different cell extracts are pooled, measured, and diluted. Aliquots of 1.2 mL are stored at −80 °C. For *K. phaffii*, a *S. cerevisiae* ^{13}C cell extract can also be used [11, 14].

4 Notes

1. It is desirable to grow cells in chemically defined media, i.e., where all the medium components are known, at moderate cell densities. Higher cell density cultures will need higher salt concentrations in the media, which may cause ion suppression effects in the electrospray ionization interface between the LC and the MS. The present protocol has been validated for cells growing on glucose [11] and glucose:methanol (80:20%, w/w) mixed feeds in chemostat cultures [14]. Since the characteristics of the cell membrane and cell wall of microorganisms may vary depending on the growth conditions, it is advisable to validate the quenching and extraction method for the new conditions, as described in [11]. Procedures for dynamic ^{13}C-labeling strategies for instationary ^{13}C-MFA can also be performed in chemostat cultures, as described in [14, 15]. Moreover, chemostat cultures are the basis for the

study of environmental perturbations, e.g., substrate pulses, where fast sampling under dynamic conditions allows characterizing the metabolic response to such perturbations [24]. A detailed protocol to perform continuous (i.e., chemostat) cultivations is presented in Chapter 33 of this book.

2. We advise to perform conventional analyses of cultivation parameters such as concentration of biomass and extracellular metabolites, as well as CO_2 and O_2 off-gas measurements. Protocols for conventional HPLC analyses of extracellular metabolites and determination of DCW (biomass concentration) are described in [23].
3. We advise to have several backup tubes in case some error/accident occurs during the fast sampling and quenching process and this needs to be repeated.
4. The sample volume is highly dependent on the biomass density, the applied analytical platform, and targeted metabolites. In this protocol, sampling 600μL of broth was sufficient. If some metabolite levels appear to be close to or below the detection limit, the sample volume and/or the concentration of the final extract could be increased. When increasing the sample volume, it is advised to increase the volume of quenching solution such that the ratio is kept the same.
5. The volume of the applied ^{13}C-labeled cell extract into the samples is highly dependent on its quality (i.e., the levels of the U-^{13}C-labelled metabolites). In this protocol, it is assumed that the addition of 120μL of ^{13}C-labeled cell extract to each sample results in comparable levels of ^{12}C and ^{13}C metabolites for most compounds in the final extract, which is beneficial for accurate quantification with IDMS. Typically, IDMS can operate over several orders of magnitude, but clearly, the noise increases when the reference (or sample) peak has only a low signal intensity.
6. Make sure to completely wipe off the sample tubes after removing them from the cryostat before weighing them to avoid an overestimation of the taken broth volume. Sample volume has to be dispensed accurately because all measurements are going to be scaled regarding this value and the amount of ^{13}C extract added to the cells (*see* Subheading 3.8).
7. After the extraction step, cell enzymes are inactivated, and the metabolites are mixed with the ^{13}C-labeled cell extract. Samples do not strictly need to be at −40 °C anymore, but they should be kept cold anyway to limit degradation.

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