Video Article

# Workflow Based on the Combination of Isotopic Tracer Experiments to Investigate Microbial Metabolism of Multiple Nutrient Sources

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## Abstract

Studies in the field of microbiology rely on the implementation of a wide range of methodologies. In particular, the development of appropriate methods substantially contributes to providing extensive knowledge of the metabolism of microorganisms growing in chemically defined media containing unique nitrogen and carbon sources. In contrast, the management through metabolism of multiple nutrient sources, despite their broad presence in natural or industrial environments, remains virtually unexplored. This situation is mainly due to the lack of suitable methodologies, which hinders investigations.

We report an experimental strategy to quantitatively and comprehensively explore how metabolism operates when a nutrient is provided as a mixture of different molecules, *i.e.*, a complex resource. Here, we describe its application for assessing the partitioning of multiple nitrogen sources through the yeast metabolic network. The workflow combines information obtained during stable isotope tracer experiments using selected $^{13}C$- or $^{15}N$-labeled substrates. It first consists of parallel and reproducible fermentations in the same medium, which includes a mixture of N-containing molecules; however,a selected nitrogen source is labeled each time. A combination of analytical procedures (HPLC, GC-MS) is implemented to assess the labeling patterns of targeted compounds and to quantify the consumption and recovery of substrates in other metabolites. An integrated analysis of the complete dataset provides an overview of the fate of consumed substrates within cells. This approach requires an accurate protocol for the collection of samples–facilitated by a robot-assisted system for online monitoring of fermentations–and the achievement of numerous time-consuming analyses. Despite these constraints, it allowed understanding, for the first time, the partitioning of multiple nitrogen sources throughout the yeast metabolic network. We elucidated the redistribution of nitrogen from more abundant sources toward other N-compounds and determined the metabolic origins of volatile molecules and proteinogenic amino acids.

## Video Link

The video component of this article can be found at https://www.jove.com/video/56393/

## Introduction

Understanding how microbial metabolism operates is a key issue for the design of efficient strategies to improve fermentation processes and modulate the production of fermentative compounds. Advances in genomics and functional genomics in these last two decades largely contributed to extending knowledge of the topology of metabolic networks in many microorganisms. Access to this information led to the development of approaches that aim for a comprehensive overview of cellular function[1]. These methodologies often rely on a model-based interpretation of measurable parameters. These experimental data include, on one hand, metabolite uptake and production rates and, on the other hand, quantitative intracellular information that is obtained from isotope tracer experiments. These data provide essential information for the deduction of the *in vivo* activity of different pathways in a defined metabolic network[2,3,4]. Currently, the available analytical techniques only enable the accurate detection of labeling patterns of molecules when using a single-element isotope and possibly when co-labeling with two isotopic elements. Moreover, under most growth conditions, the carbon source only consists of one or two-compounds. Consequently, approaches based on $^{13}C$-isotopic tracers from carbon substrates were widely and successfully applied to develop a complete understanding of carbon metabolic network operations[5,6,7,8].

In contrast, in many natural and industrial environments, the available nitrogen resource that supports microbial growth is often composed of a wide range of molecules. For example, during wine or beer fermentation, nitrogen is provided as a mixture of 18 amino acids and ammonium at variable concentrations[9]. This array of N compounds that are accessible for anabolism makes these complex media conditions greatly different from those commonly used for physiological studies, as the latter are achieved using a unique source of nitrogen, typically ammonium.

Overall, internalized nitrogen compounds may be directly incorporated into proteins or catabolized. The network structure of nitrogen metabolism in many microorganisms, including the yeast *Saccharomyces cerevisiae*, is very complex in accordance with the diversity of substrates.

 

Schematically, this system is based on the combination of the central core of nitrogen metabolism which catalyzes the interconversion of glutamine, glutamate, and α-ketoglutarate[10,11], with transaminases and deaminases. Through this network, amine groups from ammonium or other amino acids are gathered and α-keto acids released. These intermediates are also synthetized through central carbon metabolism (CCM)[12,13]. This large number of branched reactions and intermediates, involved in both the catabolism of exogenous nitrogen sources and the anabolism of proteinogenic amino acids, fulfills the anabolic requirements of the cells. The activity through these different interconnected routes also results in the excretion of metabolites. In particular, α-keto acids may be redirected through the Ehrlich pathway to produce higher alcohols and their acetate ester derivatives[14], which are essential contributors to the sensory profiles of products. Subsequently, how nitrogen metabolism operates plays a key role in biomass production and the formation of volatile molecules (aroma).

The reactions, enzymes, and genes involved in nitrogen metabolism are extensively described in the literature. However, the issue of the distribution of multiple nitrogen sources throughout a metabolic network has not yet been addressed. There are two main reasons that explain this lack of information. First, in view of the important complexity of the nitrogen metabolism network, a large amount of quantitative data is required for a complete understanding of its operation that was unavailable until now. Second, many experimental constraints and limitations of analytical methods prevented the implementation of approaches that were previously used for the elucidation of CCM function.

To overcome these problems, we chose to develop a system-level approach that is based on the reconciliation of data from a series of isotopic tracer experiments. The workflow includes:
- A set of fermentations carried out under the same environmental conditions, while a different selected nutrient source (substrate) is labeled each time.
- A combination of analytical procedures (HPLC, GC-MS) for an accurate determination, at different stages of the fermentation, of the residual concentration of labeled substrate and the concentration and the isotopic enrichment of compounds that are derived from the catabolism of the labeled molecule, including derived biomass.
- A calculation of the mass and isotopic balance for each consumed labeled molecule and a further integrated analysis of the dataset to obtain a global overview of the management of multiple nutrient sources by microorganisms through the determination of flux ratios.

To apply this methodology, attention must be paid to the reproducible behavior of the strain/microorganism between cultures. Furthermore, samples from different cultures must be taken during the same well-defined fermentation progress. In the experimental work reported in this manuscript, a robot-assisted system is used for online monitoring of fermentations to account for these constraints.

Furthermore, it is essential to choose a set of labeled substrates (compound, nature, and position of the labeling) that is appropriate to address the scientific problem of the study. Here, $^{15}$N-labeled ammonium, glutamine, and arginine were selected as the three major nitrogen sources found in grape juice. This allowed assessing the pattern of nitrogen redistribution from consumed compounds to the proteinogenic amino acids. We also aimed to investigate the fate of the carbon backbone of the consumed amino acids and their contribution to the production of volatile molecules. To meet this objective, uniformly $^{13}$C-labeled leucine, isoleucine, threonine, and valine were included in the study as amino acids that are derived from major intermediates of the Ehrlich pathway.

Overall, we quantitatively explored how yeast manages a complex nitrogen resource by redistributing exogenous nitrogen sources to fulfill its anabolic requirements throughout fermentation while additionally removing the excess of carbon precursors as volatile molecules. The experimental procedure reported in this paper can be applied to investigate other multiple nutrient sources used by any other microorganism. It appears to be an appropriate approach for the analysis of the impact of genetic background or environmental conditions on the metabolic behavior of microorganisms.

## Protocol

# 1. Fermentation and Sampling

1. **Preparation of media and fermenters**
   NOTE: All the fermentations are carried out in parallel, using the same strain and in the same chemically defined synthetic medium (SM, composition provided in **Table 1**), which includes a mixture of ammonium and amino acids as nitrogen sources[15]. For each fermentation, a single nitrogen compound is provided exclusively in a uniformly labeled $^{13}$C or $^{15}$N form (100%), while the others remain unlabeled. For each labeled nitrogen source that is used in the set of experiments (here: $^{15}NH_4$, U-$^{15}$N4-Arg, U-$^{15}$N2-Gln, U-$^{13}$C6-Leu, U-$^{13}$C5-Val, U-$^{13}$C6-Ile, U-$^{13}$C4-Thr), two fermenters are prepared. For each condition, duplicate fermentation is performed using only unlabeled molecules (7 control fermentations).
   1. For each N-source to be studied, prepare 500 mL of SM medium that contains all the nitrogen sources listed in **Table 1**, with the exception of the compound to be used in 100% labeled form.
      NOTE: The labeled molecule is added in the next steps.
   2. Pasteurize each medium in a 1 L flask (10 min, 100 °C) containing a magnetic stirring bar. Weigh the appropriate amount of labeled molecule to reach the final concentration that is reported in **Table 1** and dissolve it in the medium.
   3. Sterilize the medium using a disposable vacuum filtration system (cellulose acetate membrane, 0.22 µm). Using a sterile measuring cylinder, divide the medium between two pre-sterilized fermenters (250 mL) that contain a magnetic stirring bar and are equipped with fermentation locks to avoid the entry of oxygen but allow the release of $CO_2$.
   4. Heat the fermentation flasks to 28 °C by placing them in the incubation room for 1 night (temperature set at 28 °C).

2. **Inoculation and monitoring of fermentations**
   1. Grow *S. cerevisiae* strain in sterile tubes containing 10 mL of YPD medium at 28 °C with shaking (150 rpm) for 12 h. Then, pipet 1 mL of YPD preculture and transfer it to 10 mL of SM medium (in 15 mL sterile tubes). Incubate the culture for 12 h at 28 °C with shaking (150 rpm).

2. Under laminar flow, collect a preculture aliquot and quantify the cell population using an electronic particle counter that is fitted with a 100 µm aperture. Centrifuge the preculture (2,000 x g, 15 min, 4 °C) and suspend the pellet in an appropriate volume of sterile water to obtain a final concentration of 2.5 x $10^8$ cells/mL. Inoculate each fermenter with 1 mL of the cell suspension.
   NOTE: The robotic system used to monitor the fermentation progress is described in **Figure 1**.
3. Prepare the fermentation platform by installing the fermenters in the support guides that are properly placed on the 21-position stirring plates and set the stirring rate at 270 rpm. To start the on-line monitoring of each fermentation, launch the robot-control application, then click the "start assay" button and select the fermentation volume to be carried out (300 mL).
4. The displayed interface permits the indication of the number and the position of fermenters on the platform. To ensure this occurs, right click on the slot location and choose "enable" to activate the monitoring of the fermenter located at this position.
5. Initialize the calculation software, permanently running on the system, before starting the weight acquisition. Click on the "Initialiser" button and validate with "ok". Click on the "Start button" of the robot-control application to start the weight acquisitions.

3. **Sampling Procedure**
   NOTE: For each fermenter, samples are taken when the $CO_2$ production (value shown on-line on the computer running the calculation software) reaches the required set-point: 5, 10, 40, and 90 g/L in this study.
   1. **Sampling procedure for cultures with labeled compounds.**
      1. Centrifuge two 6 mL samples (2,000 x g, 5 min, 4 °C). Save and store the frozen supernatants in two aliquots at -80 °C. Wash the pellets twice with 5 mL distilled water and store at -80 °C for the measurement of isotopic enrichments.

   2. Sampling procedure for cultures without labeled compounds
      1. Harvest 10 mL of culture, which will be used for dry weight determination. Pellet the cells from two 10 mL samples by centrifugation (2,000 x g, 5 min, 4 °C). Wash the pellets twice with 10 mL of distilled water and store them at -80 °C for the determination of protein and amino acid content.

## 2. Quantification of Consumed Nitrogen Sources

1. **Enzymatic determination of residual ammonia concentrations**
   NOTE: The determination of ammonia concentration in supernatants is carried out using a commercial enzyme-based kit; all the reagents are provided by the manufacturer.
   1. Prepare a standard ammonia solution (61.4 mg/L) by dissolving 25 mg of precisely weighed $(NH_4)_2SO_4$ in a 100 mL volumetric flask.
   2. To fulfill the manufacturer's instructions, perform a 1:2 dilution of the samples that were taken before fermentation and at 5 g/L of $CO_2$ released. Adjust the pH of the samples to approximately 8 by adding 1 M KOH. Take note of the added volume and take it into account in the dilution factor.
   3. In 4 mL spectrophotometer cuvettes, mix 100 µL of sample (diluted if necessary), distilled water or standard ammonia solution with 2 mL of reagent 1 (0.75 mM ADP and 30 U/mL glutamate dehydrogenase in pH 7.8 buffer) and 500 µL of reagent 2 (1.3 mM NADH). Incubate for 15 min at room temperature and read NADH absorbance at 340 nm (A1).
   4. Add 500 µL of reagent 3 (60 mM α-ketoglutarate in pH 8 buffer), incubate the sample for 20 min at room temperature, and read the NADH absorbance at 340 nm (A2).
   5. Calculate ammonia concentration using:
      $C_{ammonia}$ (g/L) = 0.083 x $[(0.839 \times A1 - A2)_{sample} - (0.839 \times A1 - A2)_{distilled\ water}]$
   6. Check that the correct concentration is obtained with the standard solution.

2. **Chromatographic determination of residual amino acid concentrations**
   NOTE: The determination of amino acid concentrations in supernatants is achieved using a dedicated amino acid analysis system that is based on ion-exchange chromatography with post column derivatization of N-compounds with ninhydrin, which allows their colorimetric detection.
   1. Prepare a reference solution by adding 200 µL of a commercial mixture of neutral and acidic amino acids, 200 µL of a commercial mixture of basic amino acids, and 200 µL of 2.5 mM glutamine to 400 µL of 200 mM lithium citrate buffer, pH 2.2. This chemically defined reference solution is treated as a sample.
   2. Add 200 µL of 25% (w/v) sulfosalicylic acid solution that contains 2.5 mM norleucine (internal standard) to 800 µL of sample to remove molecules with high molecular weights. Incubate for 1 h at 4 °C, centrifuge (3,000 x g, 10 min, 4 °C) and filter through a 0.22-µm pore-size nitrocellulose membrane (syringe system).
   3. In the programmer software, click on the button "Run" to begin the liquid chromatography (LC) analyses with the analyzer equipped with a cation-exchange column (lithium form). Elute the amino acids with successive lithium buffers to create both a pH gradient and a gradient in counter-ion concentration in combination with a temperature gradient (**Table 2**).
   4. Quantify the nitrogen compounds after ninhydrin derivatization by a spectrophotometric detector at 570 nm (purple coloration: reaction between ninhydrin and amine group of amino acids) and 440 nm (yellow coloration: reaction between ninhydrin and imine group of proline).
   5. Perform a single-point internal calibration using the reference solution and norleucine as an internal standard to calculate the concentrations of amino acids in the samples using the manufacturer's software.

## 3. Quantification of Proteinogenic Amino Acids

1. **Measurement of dry cell weight**
   1. Filter 10 mL of culture through a nitrocellulose filter (pore size 0.45 µm) that is pre-weighed in an aluminum cup, using a vacuum device. Wash twice with 50 mL of distilled water.

2. Place the filter in the aluminum cup and dry in a heat oven at 105 °C for 48 h (until no further change in weight is observed) before reweighing the filter in the cup. Calculate the weight difference.
3. Calculate the average of at least 3 independent measurements to accurately determine the dry cell weight of the yeast culture.

2. **Quantification of protein content of cells**
NOTE: The quantification of the protein fraction of the cells is performed at least in triplicate using unlabeled cell pellets that were obtained as described in section 1.3.2.
1. Extract proteins by addition of 1 mL of DMSO solution (50 % v/v) to frozen pellets and incubate at 105 °C for 1 h in a dry heat oven.
2. Quantify the protein content in the DMSO extracts using the biochemical colorimetric assay that is based on the reduction by proteins of $Cu^{++}$ into $Cu^{+}$ ions that are further precipitated by bicinchoninic acid in a blue complex (BCA assay).

3. **Determination of the relative contributions of amino acids within proteins**
NOTE: The profile of proteinogenic amino acids is determined at least in triplicate from unlabeled cell pellets (1.3.2.).
1. Prepare an oxidized extract by suspending the cell pellet in 200 µL of performic acid (90% formic acid, 10% hydrogen peroxide). Incubate for 4 h at 4 °C and stop the reaction by the addition of 33.6 mg sodium sulfate.
NOTE: The oxidation step is required to convert cysteine and methionine into methionine sulfone and cysteic acid, which will be further quantified by ion-exchange chromatography. However, some amino acids (tyrosine, phenylalanine, histidine, and arginine) are denatured during oxidation treatment. Consequently, two hydrolysates (with and without oxidation) are prepared.
2. Add 800 µL of 6N HCl to cell pellets or oxidized extract and incubate the sample in a hermetically sealed glass tube for 16 h at 110 °C in a dry heat oven. Add 200 µL of 2.5 mM norleucine and remove HCl with a stream of nitrogen. Wash (resuspending the dried extract and then removing the liquid with a nitrogen stream) twice with 800 µL of distilled water and then with 800 µL of ethanol. Take up in 800 µL of 200 mM lithium acetate buffer, pH 2.2.
NOTE: Attention should be paid to the incubation time for the hydrolysis as some amino acids are not stable under acidic conditions. The tryptophan fraction in protein is estimated from data found in the literature[16], as this amino acid is entirely denatured during HCl hydrolysis.
3. Prepare a hydrolysate standard for the single-point internal calibration. Add 160 µL of a commercial solution of hydrolyzed amino acids to 840 µL of 200 mM lithium acetate buffer, pH 2.2, that contains 625 µM methionine sulfone, 625 µM cysteic acid, and 625 µM norleucine.
4. Determine the relative concentrations of amino acids within proteins using the chromatographic method described in section 2.2.

4. **Calculations**
1. Calculate the weight percentage of each amino acid in proteins by dividing the measured amount of each amino acid (mg/L) by the total amount of amino acid that was measured in the protein extract (sum in mg/L).
2. Multiply this percentage by the concentration of proteins in the culture (mg/L), *i.e.*, the product between the protein content of the biomass and the dry weight, to assess the concentration of each proteinogenic amino acid in the culture (mg/L).

## 4. Measurement of Isotopic Enrichment of Proteinogenic Amino Acids

NOTE: For the measurement of isotopic enrichment of proteinogenic amino acids, use the labeled cell pellets. Three different agents are used for the derivatization step to quantify the isotopic enrichment of amino acids. The intensities of cluster ions are measured to estimate the labeling patterns of the amino acids. The signal from each cluster ion corresponds to the abundance of the mass isotopomers ($m_0$ = without labeling, $m_{+1}$ = 1 labeled atom, …) of an amino acid fragment. An example of a chromatogram that is obtained after the DMADMF procedure is provided in **Figure 2**.

1. **Biomass hydrolysis**
1. Hydrolyze the cell pellets (corresponding to 1-2 mg of dried biomass) by adding 1.2 mL of 6 M HCl and incubating the sample for 16 h at 105 °C in tightly closed glass tubes in a dry heat oven.
2. Add 1.2 mL of distilled water and centrifuge at 3,000 x *g* for 5 min to remove cellular debris. Distribute the supernatant into six 400 µL fractions in open glass tubes. Dry the fractions in a heat oven at 105 °C until they reach the consistency of syrup (4-5 h).

2. **Ethylchloroformate (ECF) derivatization**
1. Dissolve the dried hydrolysate in 200 µL of 20 mM HCl; then, add 133 µL of pyridine:ethanol (1:4). Add 50 µL of ECF to derivatize the amino acids and wait until all $CO_2$ has been released. Transfer the mixture to centrifuge tubes that contain 500 µL of dichloromethane to extract the derivatized compounds.
2. Vortex the tubes for 10 s and centrifuge them for 4 min at 10,000 x g; collect the lower organic phase with a Pasteur pipet and transfer to GC vials that contain conical glass inserts so that the samples may be directly injected into the GC/MS instrument.

3. **(N,N)-Dimethylformamide dimethyl acetal (DMFDMA) derivatization.**
1. Dissolve the dried hydrolysate in 50 µL of methanol and 200 µL of acetonitrile. Add 300 µL of DMFDMA. Vortex the tube and transfer the samples to GC autosampler vials that contain conical glass inserts.

4. **N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) derivatization**
1. Suspend the hydrolysate in 200 µL of acetonitrile. Add 200 µL of BSTFA, hermetically close the glass tube and incubate for 4 h at 135 °C before transferring the extract directly to GC vials.

5. **GC-MS analysis**
1. Analyze samples with a gas chromatograph that is equipped with an autosampler injector and is coupled to a mass spectrometer.
1. Use instrument-specific software to control the instrument and analyze the chromatograms. In the "Sequence" menu, click the "Sample Log Table" to create the sample list, and click the "Run" button to start the injections.

NOTE: The gas chromatograph is fitted with a 30 m x 0.25 mm apolar silica capillary column with a 0.15 µm film thickness. Set the mass spectrometer quadrupole temperature at 150 °C and hold the transfer line at 250 °C for all the analyses. Three analytical programs, each one specific to each derivatization agent, are used.

2. **ECF derivatives:** Use helium as the mobile phase with a flow of 1.2 mL/min. Set the temperature of the inlet at 230 °C and that of the source at 250 °C. Program the autosampler to inject 1 µL of samples with a split ratio of 3:1. Run the analyses, gradually increasing the oven temperature as follows: 130 °C for 3 min; gradient of 15 °C/min to 260 °C; maintain temperature at 260 °C for 20 min.
3. **DMFDMA derivatives:** Use helium as the mobile phase with a constant flow of 1.2 mL/min. Set the temperature of the inlet at 230 °C and that of the source at 250 °C. Program the autosampler to inject 1 µL of samples with a split ratio of 3:1. Run the analyses, gradually increasing the oven temperature as follows: 60 °C for 1 min; gradient of 20 °C/min to 130 °C; second gradient of 4 °C/min to 260 °C; maintain temperature at 260 °C for 10 min.
4. **BSTFA derivatives:** Use helium as the mobile phase with a constant flow of 1.2 mL/min. Set the temperature of the inlet at 275 °C and that of the source at 300 °C. Run the analyses (injection: 1 µL), gradually increasing the oven temperature as follows: 110 °C for 1 min; first gradient of 2 °C/min to 154 °C; second gradient of 5 °C/min to 300 °C; maintain temperature at 300 °C for 10 min.
5. **Detection procedure:** For each mode of derivatization, inject a sample (1 µL) in SCAN mode with positive electron impact ionization at 70 eV and take note of the retention time of each amino acid.
6. Use these values to define the time windows throughout the chromatogram and for the different selected ions, which are characteristic of each amino acid and listed in **Table 3**; these values should be included for each amino acid. Include this information in the SIM detection program and run the analyses in SIM mode with positive electron impact ionization at 70 eV.

2. Collect the outcomes of the analyses; namely, for each amino acid, record a cluster of intensities that correspond to its different mass isotopomers. Process the data using the dedicatedsoftware[17] to correct for natural labeling and calculate the isotopic enrichment of the proteinogenic amino acids (defined as the labeled fraction of an amino acid with respect to its total amount in the protein samples).
NOTE: The isotopic enrichment of a molecule (I.E.), expressed as a percentage, is calculated dividing the sum of the corrected intensities of the mass isotopomers with labeling ($m_1$, $m_2$, …$m_n$) by the sum of the corrected intensities of all the mass isotopomers ($m_0$, $m_1$, $m_2$,… $m_n$):
I. E. = ($m_1$ + $m_2$ + …+ $m_n$) / ($m_0$ + $m_1$+ $m_2$ + …+ $m_n$)

## 5. Quantification and Isotopic Enrichment of Volatile Compounds

1. **Extraction of labeled volatile compounds**
   1. Add 10 µL of deuterated internal standards to 5 mL of supernatant (final concentration of deuterated compounds: 100 µg/L) in a 15 mL glass tube. Add 1 mL of dichloromethane, tightly close the tubes and shake them on a rocking platform for 20 min. Centrifuge for 5 min at 3,000 x g and collect the organic lower phase in a 15 mL glass tube. Repeat the dichloromethane extraction.
   2. Dry the organic extract over 500 mg of anhydrous sodium sulfate and collect the liquid phase with a Pasteur pipet. Concentrate the extract by a factor of four under nitrogen flux and transfer it to a GC autosampler vial.

2. **GC-MS quantification of volatile compounds**
   1. Equip the gas chromatograph with a 30 m x 0.25 mm fused silica capillary column with 0.25 µm film thickness and apply a constant helium flow of 1.0 mL/min. Hold the injector and the transfer line at 250 °C.
   2. Inject 2 µL of sample with a split ratio of 10:1 and separate the extracted volatile molecules using the following oven temperature profile: hold the temperature for 3 min at 40 °C, increase it by 4 °C/min up to 220 °C and then hold the oven at 220 °C for 20 min.
   3. Detect the compounds using a mass spectrometer with its source temperature set at 230 °C and its mass spectrometer quadrupole temperature set at 150 °C. Record the mass spectra in Selected Ion Monitoring (SIM) mode with positive electron impact ionization at 70 eV and using the ion clusters specific to the volatile compounds that are reported in **Table 4**.
   4. Use an external 7-point calibration to quantify the concentrations of volatile molecules from the sums of the intensities of the corresponding ion cluster. Prepare stock solutions of each compound (10 g/L) in 100% ethanol. Then, prepare standard solutions for each class of volatile molecules (ethyl esters, acetates, alcohols, and acids) by mixing stock solutions. Finally, dilute different amounts of the standard solutions in a 12% hydroalcoholic solution containing 5 g/L tartaric acid with the pH adjusted to 3.3 to prepare calibration solutions.
   5. In parallel, correct for the natural labeling of the intensities of each ion cluster and calculate the isotopic enrichment of volatile compounds, which is defined as the labeled fraction of the molecules and is expressed as a percentage using the dedicated software[17].

## 6. Calculations for an Integrated Analysis of the Dataset

1. **Collection of the raw data**
   1. Using the spreadsheets shown in **Tables 5, 6, 7,** and **8,** enter the raw data values that correspond to the concentration of extracellular amino acids, cell dry weight, protein content of cells, concentration of volatile molecules, and isotopic enrichment of proteinogenic amino acids and volatile molecules.
   NOTE: The data shown in tables are expressed in mM. All the results are also expressed in mg/L by multiplying the values in mM by the molecular weight of each molecule or in mg N/L by multiplying the millimolar concentration of a proteinogenic amino acid by the atomic mass of nitrogen (14 u) and by the number of nitrogen atoms that are provided by the catabolism of this molecule.
   2. Calculate the mass percentage of each amino acid in proteins by dividing its amount in mg/L in the hydrolysate by the total amount of amino acids (their sum in mg/L).
   3. Calculate means, standard deviations, and standard errors of the mean from the data that were obtained in the independent experiments.

4. Calculate the proteinogenic concentration (mg/L) for each amino acid by multiplying the percentage of this amino acid in proteins (mg aa/g proteins) by the protein fraction in the biomass (g proteins/g DW) and the dry weight content (biomass production) in the medium (g DW/L).

2. **$^{15}$N isotopic tracer experiments**
   1. Using the spreadsheets that are presented in **Table 9**, calculate the labeled and unlabeled fractions of nitrogen that are present in the proteinogenic amino acids (expressed in mg N/L) from their total concentrations (expressed in mg N/L) and their isotopic enrichments. For each amino acid, the labeled fraction corresponds to the product between its total concentration and its isotopic enrichment, and the unlabeled part is the difference between the total and the labeled amounts.
   2. Then, determine the fraction of total nitrogen in proteins that is contained in the proteinogenic amino acids quantified in this study by summing the total amount of Ala, Gly, Val, Asp, Phe, Leu, Ile, Thr, Ser, Pro, Lys, His, Glu and Arg (in mg N/L) and dividing the total amount of nitrogen contained in proteins by this sum.
   3. Calculate the nitrogen provided by arginine, glutamine or ammonium that was recovered in the proteinogenic acids quantified in this study by summing the labeled fraction (in mg N/L) of proteinogenic amino acids that were quantified in the study (Ala, Gly, Val, Asp, Phe, Leu, Ile, Thr, Ser, Pro, Lys, His, Glu, and Arg) during experiments in the presence of $^{15}$N-labeled arginine, glutamine, or ammonium and dividing this labeled-nitrogen fraction by the total amount of nitrogen in the quantified proteinogenic amino acids.
   4. Assess the contribution of the 3 most abundant amino acids to the intracellular pool of nitrogen used for *de novo* biosynthesis by combining data from the $^{13}$C and $^{15}$N isotope tracer experiments (spreadsheet in **Table 7**).
   5. From the total amount (expressed in mM) of proteinogenic Val, Leu, Ile, or Thr, deduct the part derived from the direct incorporation of consumed compounds ($^{13}$C experiments) and the part that was *de novo* synthetized using nitrogen from the 3 major sources in order to assess the fraction that was *de novo* synthetized using nitrogen from the other amino acids.
   6. Then, calculate the ratio of the amount of amino acid *de novo* synthetized using nitrogen provided by Gln, Arg, and $NH_4^+$ (sum expressed in mM) to the total amount of proteinogenic amino acids (in mM) to quantify the contribution of arginine, glutamine, and ammonium to the intracellular nitrogen pool.

3. **$^{13}$C isotopic tracer experiments**
   1. Calculate the labeled and unlabeled fractions of the proteinogenic amino acids from concentrations expressed in mM and isotopic enrichments of proteinogenic amino acids that were obtained during experiments in the presence of $^{13}$C-labeled Leu, Val, Ile or Thr. (see 6.2.1, **Table 10**).
   2. Calculate the labeled and unlabeled fractions of volatile compounds from concentrations expressed in mM and isotopic enrichments of volatile compounds that were obtained during experiments in the presence of $^{13}$C-labeled Leu, Val, Ile, or Thr (**Table 10**).

## Representative Results

**Figure 3** presents a schematic diagram of the workflow that was implemented to investigate the management by yeast of the multiple nitrogen sources that are found during wine fermentation.
For different points of sampling, the biological parameters–growth characteristics, nitrogen consumption patterns, and the profile of proteinogenic amino acids–show a high reproducibility among fermentations (**Figure 4**). This consistency validates the relevance of the approach based on the combination of data that was generated during a set of 14 independent fermentations.

**Figure 5** shows a comprehensive overview of the redistribution of nitrogen from the sources that are available in the grape juice to all the proteinogenic amino acids. This analysis is mainly supported by the results that were obtained from experiments carried out in the presence of $^{15}$N-labeled compounds. Combined with the determination of the mass and isotopic balances, the measurement of the isotopic enrichment of proteinogenic amino acids provided the first quantification of the contribution of nitrogen-originated arginine, glutamine, ammonium, and other sources to the amine groups of each of these compounds. The important redistribution of nitrogen that is underlined here reflects the substantial role of *de novo* synthesis of amino acids in sustaining yeast growth.

Furthermore, this study, comparing the amounts of labeled compounds in proteins with their consumption, allows the fraction of consumed N-containing molecules that are directly incorporated into proteins when they enter the cells to be assessed. Proteinogenic amino acids are differentiated according to their level of direct incorporation in the biomass; some of them are exclusively (Asp, Glu) or more than 80% *de novo* synthetized, while only small amounts of other compounds are generated by *de novo* synthesis. Interestingly, this last group includes lysine and histidine, which are the only amino acids in which all the consumed sources are directly incorporated.

**Figure 5B** also presents, for some amino acids, a comparison between the amount of consumed amino acid that is directly recovered into the biomass, calculated from data obtained in $^{15}$N-labeling experiments and measured experimentally in $^{13}$C-labeling experiments. The small differences between these values show the reliability and the robustness of the approach that was implemented in this study.

**Figure 6** shows an example of quantitative partitioning (ratios) of fluxes through metabolic pathways that was obtained by implementing the workflow reported in this paper. This map was drawn by combining information from isotopic tracer experiments using $^{15}$N- and $^{13}$C-labeled substrates and describes the partitioning of the consumed aliphatic amino acids in the metabolic network that is involved in valine and leucine biosynthesis in yeast (for calculations, refer to **Table 1** and **Table 2**). By measuring the production and isotopic enrichment of both proteinogenic amino acids and volatile compounds, we assessed the amount of these compounds that were synthetized from the carbon backbones of consumed U-C$^{13}$-valine and U-C$^{13}$-leucine, which corresponds to the labeled fraction of the compounds. Subsequently, we were able to determine the contribution of CCM to their formation (the unlabeled fraction of the compounds). Carbon mass balances set up using the workflow and the calculation procedure that is proposed here show that more than 96% of the consumed valine and leucine are recovered in their conversion products, namely, proteinogenic leucine and valine and volatile higher alcohols. This finding confirms the suitability of the reported approach for quantitative studies of metabolism. The integrated and comprehensive analysis of the dataset offers new insights in the fate of consumed amino acids. Surprisingly, a substantial fraction of valine and leucine are catabolized despite a considerable imbalance between the availability of these compounds in the medium and their content in biomass. However, the fraction of exogenous N-compounds directly incorporated into the proteins depends on the nature of the amino acid. Another key point concerns the metabolic origin of proteinogenic amino acids and volatile molecules. The analysis of the $^{13}$C-labeling pattern of proteinogenic leucine and valine reveals that the carbon skeleton of these amino acids mainly comes from precursors that were synthetized through the CCM. In line with this observation, the low incorporation of labeling in isobutanol and isoamyl alcohol demonstrates the very limited involvement of the catabolism of valine and leucine that were consumed by yeast in the formation of these higher alcohols.

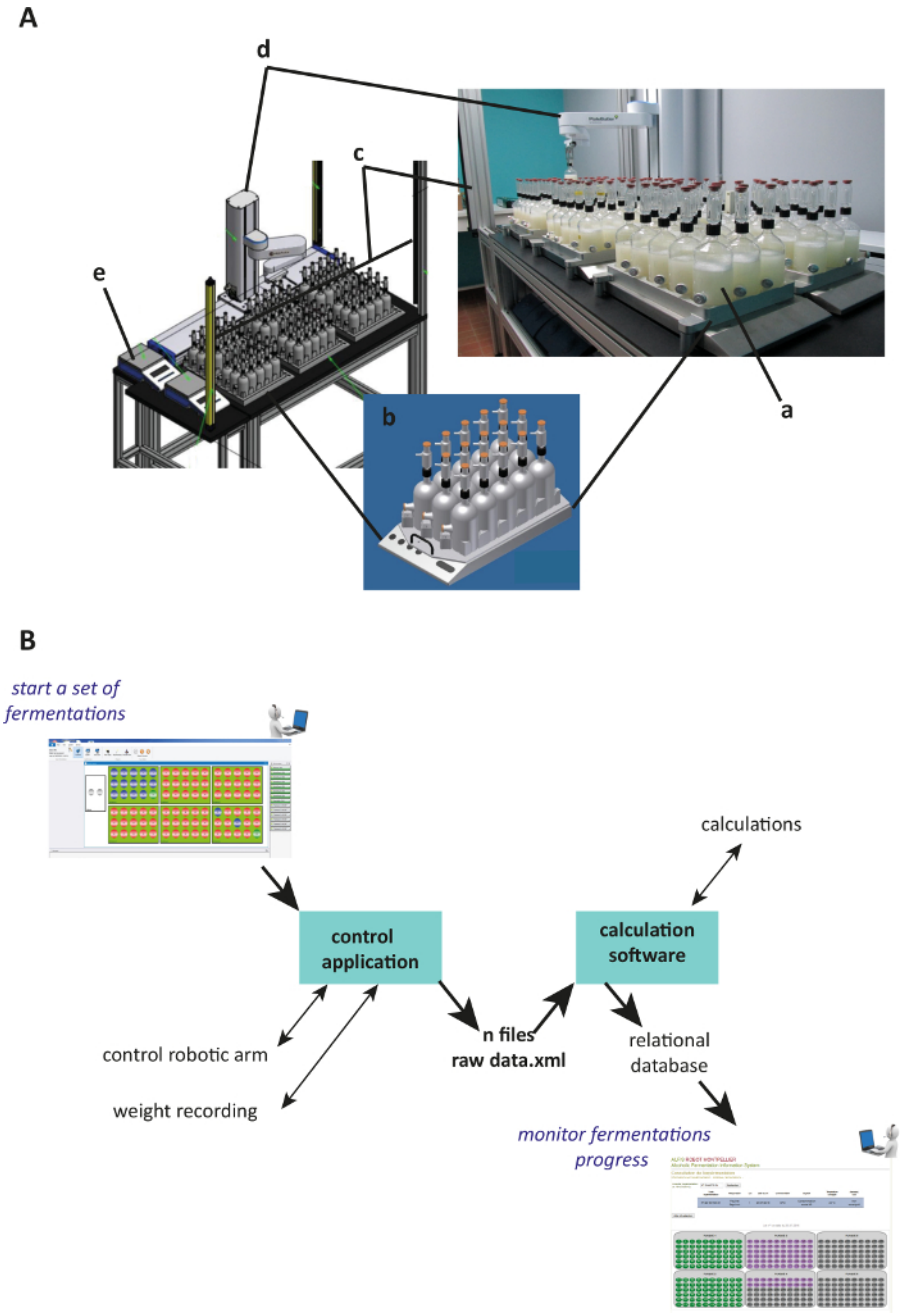


**Figure 1. Automated robotic system for monitoring high-throughput fermentations.** (**A**) Robotic platform. Fermenters (a) are placed in support guides on magnetic stirring plates (b). A safety light curtain (c) protects the users. A robotic arm (d) moves the fermenters successively from their location on one of the 6 stirring plates to a precision balance (e) on the left of the worktable, to measure the weight every 4 hours. The robotic platform is located in a temperature-controlled room. (**B**) Schematic representation of the software architecture. A graphical interface enables the users to define the experimental settings. This information is transmitted to the control application that controls the robotic arm and records the different weight in different raw data.xlm files (1 file/fermentation). The calculation software then collects the xlm files and calculates, for each time point, the amount of $CO_2$ that is released (expressed in g/L), which is proportional to the amount of sugars that have been consumed at this time, and the fermentation rate, which corresponds to the rate of $CO_2$ production, in g $CO_2$/L/h (proportional to the rate of sugar consumption). Data are stored in a relational database and visualized using a devoted graphical interface. Please click here to view a larger version of this figure.

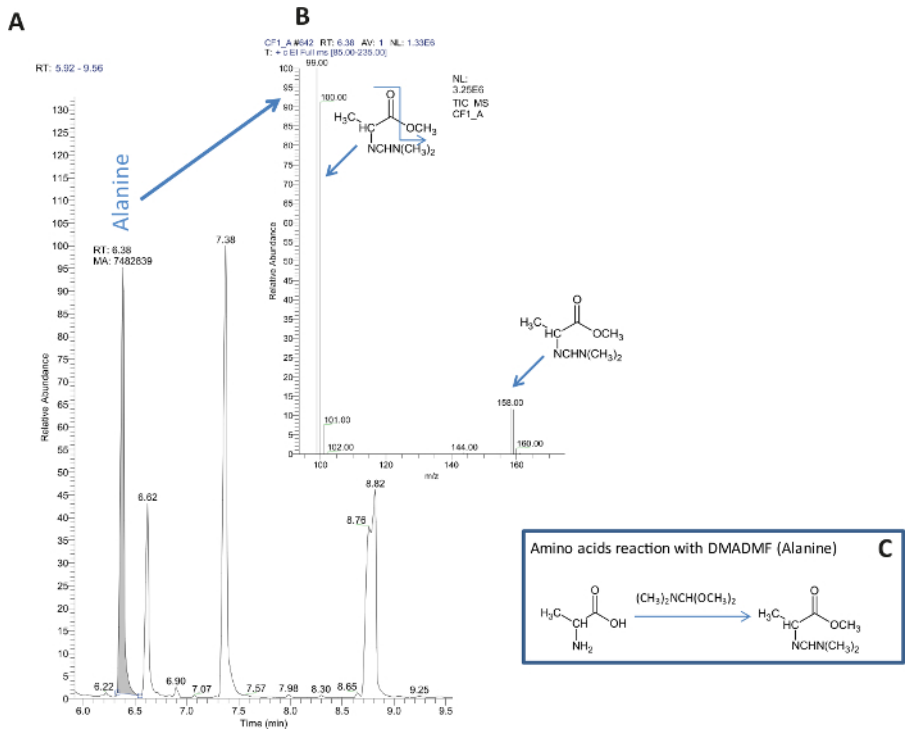


**Figure 2. GC-MS analysis of proteinogenic amino acids: example of alanine.** (**A**) Chromatogram obtained after derivatization of proteinogenic amino acids using DMFDMA. (**B**) Mass spectra of derivatized alanine. Two main peaks that correspond to fragments with $m_0/z$ = 99 and $m_0/z$ = 158. (**C**) Derivatization reaction of alanine with DMFDMA. Please click here to view a larger version of this figure.

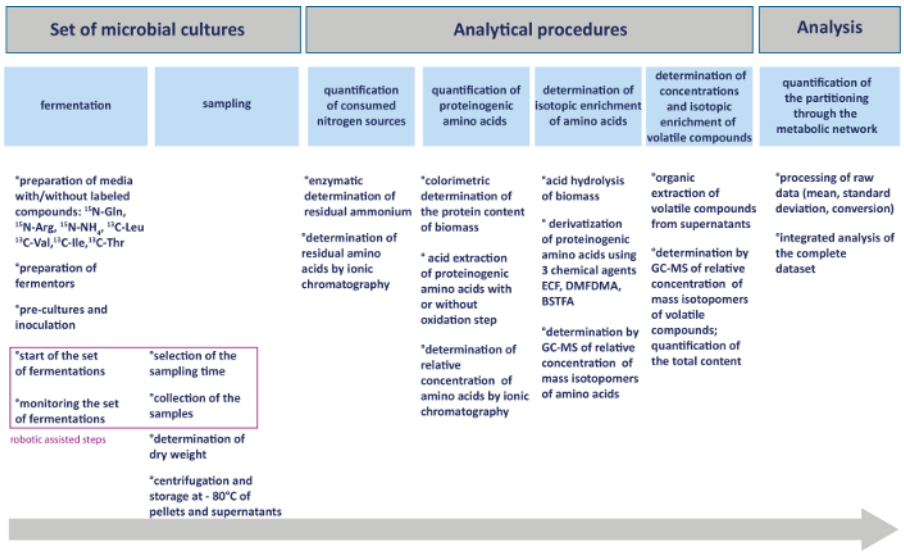


**Figure 3. Workflow for the quantitative analysis of the metabolism of multiple nitrogen sources by yeast.** This process includes (i) a set of 28 fermentations (7 nitrogen sources and fermentations with or without nitrogen compounds in duplicate); (ii) an analytical part that involves different procedures of extraction and derivatization of molecules and HPLC or GM-MS analyses and (iii) processing of raw data and an integrated analysis of the datasets. Please click here to view a larger version of this figure.

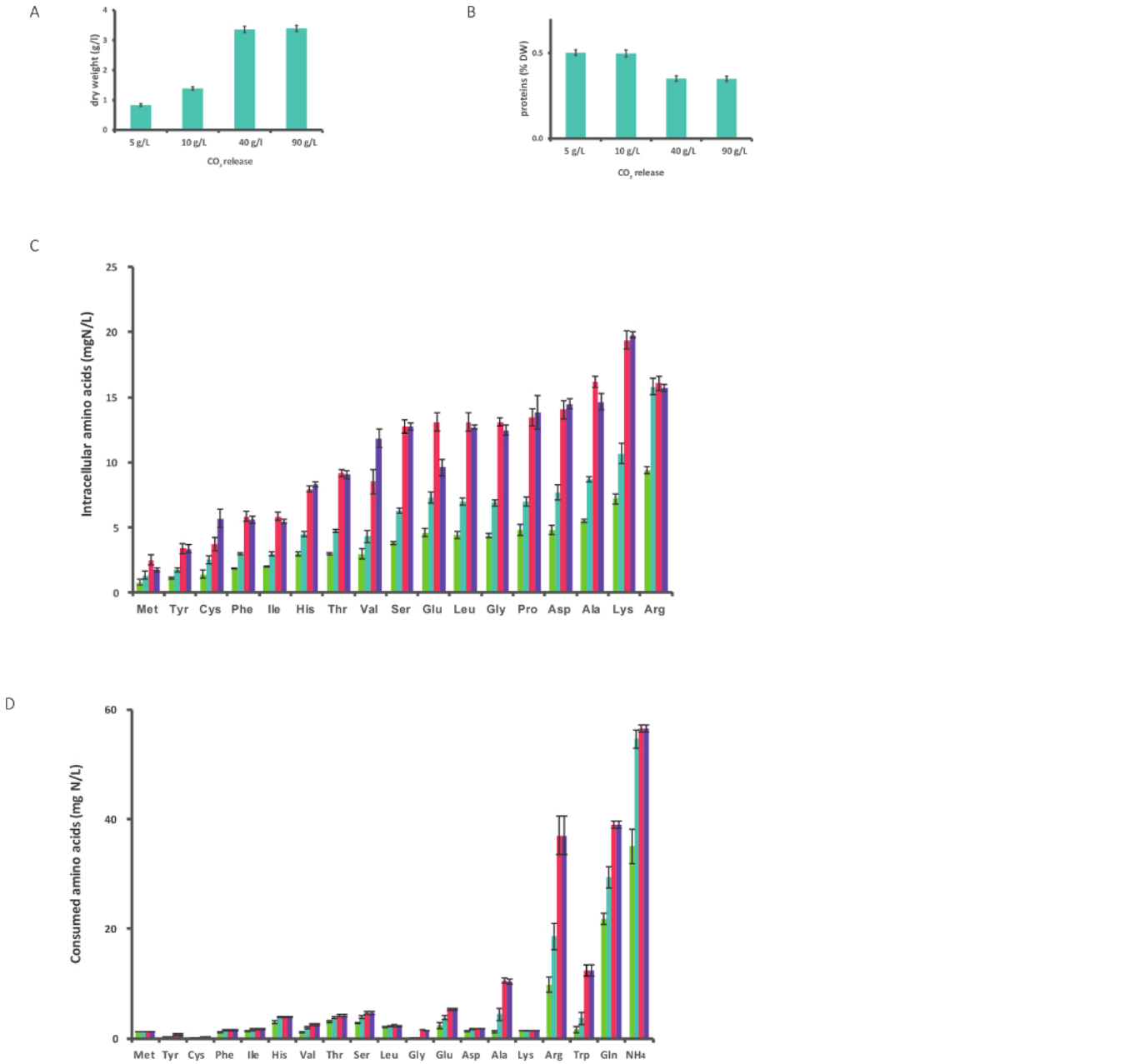


**Figure 4. Reproducibility of biological parameters from a set of independent fermentations.** (**A-B**) Mean values and standard deviations of dry weight and protein content that were obtained from the 14 independent fermentations carried out using unlabeled compounds. (**C-D**) Mean values and standard deviations of proteinogenic and consumed amino acids that were measured during 14 independent fermentations carried out using unlabeled compounds. $CO_2$ production: 5 (green), 10 (blue), 40 (pink) and 90 (purple) g/L. Please click here to view a larger version of this figure.

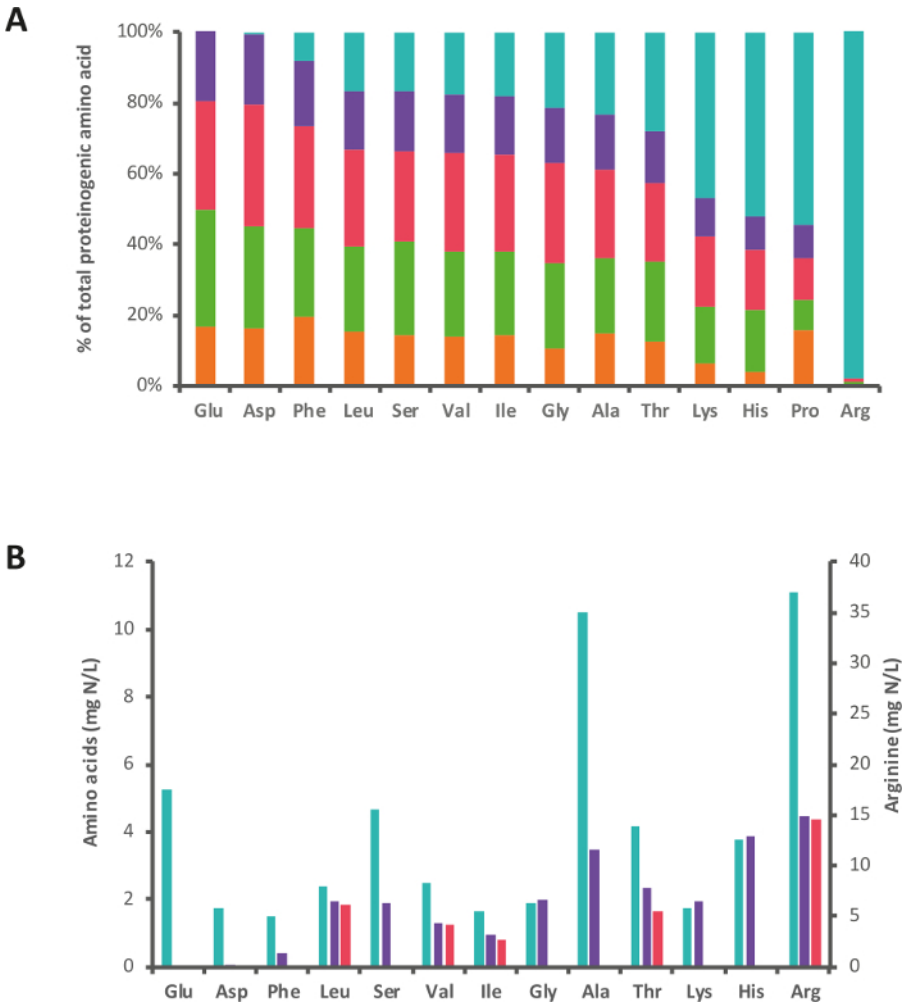


**Figure 5. Redistribution of nitrogen from the three major nitrogen sources to proteinogenic amino acids during fermentation.** (**A**) Metabolic origin of proteinogenic amino acids: direct incorporation of consumed counterpart (blue) and *de novo* synthesis using nitrogen provided by consumed arginine (orange), glutamine (green), ammonium (pink) or other amino acids (purple). Expressed as a percentage of the total amount of each proteinogenic amino acid. (**B**) Comparison between the amount of consumed amino acid (blue) and the part of the consumed amino acid that is directly recovered in proteins, calculated from the data that were obtained in $^{15}N$ tracer experiments (purple) or experimentally measured during fermentation with $^{13}C$-labeled molecules (pink). Please click here to view a larger version of this figure.

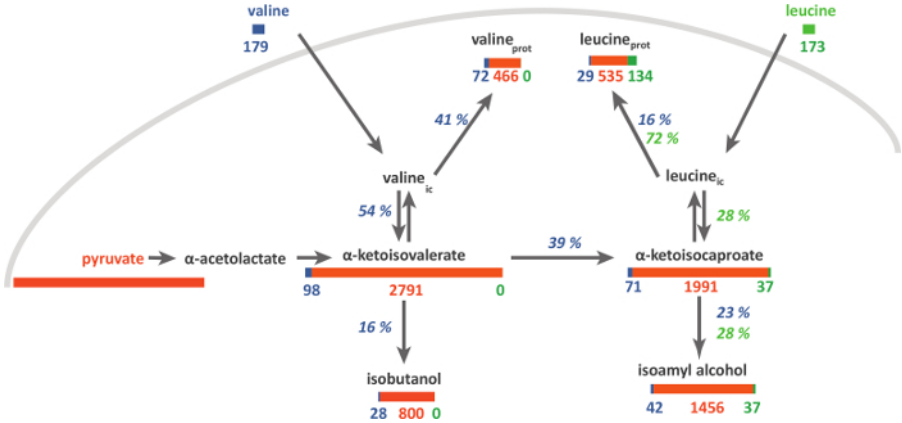


**Figure 6. Quantitative analysis of valine and leucine metabolism.** Partitioning of consumed leucine (green) and valine (blue) through the metabolic network when 40 g/L of $CO_2$ are produced. The bars are proportional to the amount of each compound and are expressed in µM, including the fraction that was synthetized from central carbon metabolism (orange). Values in the regular font: amount in µM; values in the italic font: percentage of consumed valine (blue) or leucine (green) that was catabolized through the pathway. The calculations are provided in **Table 1** and **Table 2**. Please click here to view a larger version of this figure.

| Compounds | Amount per liter |
|---|---|
| Glucose $C_6H_{12}O_6$ | 240 g |
| Malic acid $C_4H_6O_5$ | 6 g |
| Citric acid $C_6H_8O_7$ | 6 g |
| Potassium phosphate $KH_2PO_4$ | 0.75g |
| Potassium sulfate K2SO4 | 0.5 g |
| Magnesium sulfate $MgSO_4$, $7H_2O$ | 0.25 g |
| Calcium chloride $CaCl_2$, $2H_2O$ | 0.155g |
| Sodium chloride NaCl | 0.2g |
| Myo-inositol | 20 mg |
| Calcium pantothenate | 1.5 mg |
| Thiamin hydrochloride | 0.223 mg |
| Nicotinic acid | 2 mg |
| Pyridoxine | 0.25 mg |
| Biotin | 0.003 |
| $MnSO_4 \cdot H_2O$ | 4 mg |
| $ZnSO_4 \cdot 7H_2O$ | 4 mg |
| $CuSO_4 \cdot 5H_2O$ | 1 mg |
| $CoCl_2 \cdot 6H_2O$ | 0.4 mg |
| $H_3BO_3$ | 1 mg |
| $(NH_4)_6Mo_7O_{24}$ | 1 mg |
| Ergosterol | 3.75 mg |
| Oleic acid | 1.25 µL |
| Tween 80 | 125 µL |
| Tyrosine | 8.6 mg |
| Tryptophan | 84.4 mg |
| Isoleucine | 15.4 mg |
| Aspartate | 20.9 mg |
| Glutamate | 56.7 mg |
| Arginine | 176.2 mg |
| Leucine | 22.8 mg |
| Threonine | 35.7 mg |
| Glycine | 8.6 mg |
| Glutamine | 237.8 mg |
| Alanine | 68.4 mg |
| Valine | 20.9 mg |
| Methionine | 14.8 mg |
| Phenylalanine | 17.9 mg |
| Serine | 37.0 mg |
| Histidine | 15.4mg |
| Lysine | 8.0 mg |
| Cysteine | 6.2 mg |
| Proline | 288.3 mg |
| Ammonia chloride $NH_4Cl$ | 220 mg |

| |
|---|
| The pH of the medium was adjusted to 3.3 with NaOH 10 M. |

**Table 1. Composition of the synthetic medium used in this study.** This chemically defined medium mimics the composition of grape juice.

| | **Elution buffer** | **Temperature** |
|---|---|---|
| From 0 to 6 min | Lithium citrate, 200 mM, pH 2.8 | 32 °C |
| From 6 to 38 min | Lithium citrate, 300 mM, pH 3 | 32 °C |
| From 38 to 57 min | Lithium citrate, 500 mM, pH 3.15 | 64.5 °C |
| From 57 to 83 min | Lithium citrate, 900 mM, pH 3.5 | 75 °C |
| From 83 to 120 min | Lithium citrate, 1650 mM, pH 3.55 | 75 °C |
| From 120 to 130 min | Lithium hydroxyde, 300 mM | |

**Table 2. Conditions used for the separation of amino acids by ion-exchange chromatography.** Elution buffers with increased salt concentrations are used successively in combination with a temperature gradient to enable the separation of the amino acids.

| Amino acids | Derivatizing reagent | RT (min) | Ion clusters (m/z) |
|---|---|---|---|
| Alanine | ECF[a] | 3.86 | 116, 117, 118, 119 |
| | DMFDMA[b] | 6.37 | 99, 100, 101, 102 |
| | DMFDMA[b] | 6.37 | 158, 159, 160, 161 |
| Glycine | ECF[a] | 4.19 | 102, 103, 104 |
| | ECF[a] | 4.19 | 175, 176, 177 |
| | DMFDMA[b] | 6.61 | 85, 86, 87 |
| | DMFDMA[b] | 6.61 | 144, 145, 146 |
| Valine | ECF[a] | 4.97 | 144, 145, 146, 147, 148, 149 |
| | DMFDMA[b] | 7.37 | 127, 128, 129, 130, 131, 132 |
| | DMFDMA[b] | 7.37 | 143, 144, 145, 146, 147, 148 |
| | DMFDMA[b] | 7.37 | 186, 187, 188, 189, 190, 191 |
| Leucine | ECF[a] | 5.67 | 158, 159, 160, 161, 162, 163, 164 |
| Isoleucine | ECF[a] | 5.85 | 158, 159, 160, 161, 162, 163, 165 |
| Threonine | ECF[a] | 6.48 | 146, 147, 148, 149, 150 |
| | ECF[a] | 6.48 | 175, 176, 177, 178, 179 |
| Serine | ECF[a] | 6.53 | 132, 133, 134, 135 |
| | ECF[a] | 6.53 | 175, 176, 177, 178 |
| Proline | ECF[a] | 6.83 | 142, 143, 144, 145, 146, 147 |
| Aspartate | ECF[a] | 7.89 | 188, 189, 190, 191, 192 |
| | DMFDMA[b] | 11.77 | 115, 116, 117, 118, 119 |
| | DMFDMA[b] | 11.77 | 216, 217, 218, 219, 220 |
| Glutamate | ECF[a] | 8.81 | 202, 203, 204, 205, 206, 207 |
| | DMFDMA[b] | 12.75 | 111, 112, 113, 114, 115, 116 |
| | DMFDMA[b] | 12.75 | 143, 144, 145, 146, 147, 148 |
| | DMFDMA[b] | 12.75 | 230, 231, 232, 233, 234, 235 |
| Phenylalanine | ECF[a] | 9.53 | 192, 193, 194, 195, 196, 197, 198, 199, 200, 201 |
| | DMFDMA[b] | 13.67 | 143, 144, 145, 146, 147, 148, 149, 150, 151, 152 |
| Lysine | ECF[a] | 11.95 | 156, 157, 158, 159, 160, 161, 162 |
| Histidine | ECF[a] | 12.54 | 327, 328, 329, 330, 331, 332, 333 |
| Arginine | BSTFA[c] | 18.8 | 174, 175, 176, 177, 178, 179 |

[a]ECF, ethyl chloroformate ; [b]DMFDMA, (N,N)-dimethylformamide dibutyl acetal ; [c]BSTFA, (N,O-bis(trimethylsilyl)trifluoroacetamide).

**Table 3. Analytical parameters used for the determination of isotopic enrichments of amino acids using selected ion monitoring (SIM) mode.** This table summarizes the chemical agents used for derivatization, the retention time of derivatized compounds and the cluster of ions obtained in MS (mode Electron Impact) for each amino acid.

| Amino acids | Derivatizing reagent | RT (min) | Ion clusters (m/z) |
|---|---|---|---|
| Alanine | ECF[a] | 3.86 | 116, 117, 118, 119 |
| | DMFDMA[b] | 6.37 | 99, 100, 101, 102 |
| | DMFDMA[b] | 6.37 | 158, 159, 160, 161 |
| Glycine | ECF[a] | 4.19 | 102, 103, 104 |
| | ECF[a] | 4.19 | 175, 176, 177 |
| | DMFDMA[b] | 6.61 | 85, 86, 87 |
| | DMFDMA[b] | 6.61 | 144, 145, 146 |
| Valine | ECF[a] | 4.97 | 144, 145, 146, 147, 148, 149 |
| | DMFDMA[b] | 7.37 | 127, 128, 129, 130, 131, 132 |
| | DMFDMA[b] | 7.37 | 143, 144, 145, 146, 147, 148 |
| | DMFDMA[b] | 7.37 | 186, 187, 188, 189, 190, 191 |
| Leucine | ECF[a] | 5.67 | 158, 159, 160, 161, 162, 163, 164 |
| Isoleucine | ECF[a] | 5.85 | 158, 159, 160, 161, 162, 163, 165 |
| Threonin | ECF[a] | 6.48 | 146, 147, 148, 149, 150 |
| | ECF[a] | 6.48 | 175, 176, 177, 178, 179 |
| Serine | ECF[a] | 6.53 | 132, 133, 134, 135 |
| | ECF[a] | 6.53 | 175, 176, 177, 178 |
| Proline | ECF[a] | 6.83 | 142, 143, 144, 145, 146, 147 |
| Aspartate | ECF[a] | 7.89 | 188, 189, 190, 191, 192 |
| | DMFDMA[b] | 11.77 | 115, 116, 117, 118, 119 |
| | DMFDMA[b] | 11.77 | 216, 217, 218, 219, 220 |
| Glutamate | ECF[a] | 8.81 | 202, 203, 204, 205, 206, 207 |
| | DMFDMA[b] | 12.75 | 111, 112, 113, 114, 115, 116 |
| | DMFDMA[b] | 12.75 | 143, 144, 145, 146, 147, 148 |
| | DMFDMA[b] | 12.75 | 230, 231, 232, 233, 234, 235 |
| Phenylalanine | ECF[a] | 9.53 | 192, 193, 194, 195, 196, 197, 198, 199, 200, 201 |
| | DMFDMA[b] | 13.67 | 143, 144, 145, 146, 147, 148, 149, 150, 151, 152 |
| Lysine | ECF[a] | 11.95 | 156, 157, 158, 159, 160, 161, 162 |
| Histidine | ECF[a] | 12.54 | 327, 328, 329, 330, 331, 332, 333 |
| Arginine | BSTFA[c] | 18.8 | 174, 175, 176, 177, 178, 179 |

**Table 4. Analytical parameters used for the determination of isotopic enrichments and quantification of volatile compounds using selected ion monitoring (SIM) mode.** This table summarizes the retention time and the cluster of ions obtained in MS (mode Electron Impact) for each volatile compound.

**Table 5. Processing of raw data obtained from the quantification of residual amino acids** Dataset contains data from measurements of initial and residual amino acids. These values are used to calculate the amount of consumed amino acid (by subtraction). Means and standard deviations are calculated for further calculations. Please click here to download this table.

**Table 6. Processing of raw data obtained from the quantification of proteinogenic amino acids.** This dataset contains data on the composition in amino acids of biomass hydrolysates (**A**) and the fraction of proteins in biomass (**B**). These values are used to assess the mass percentage of each amino acid in proteins (**C**). Means and standard deviations are calculated for further calculations. Please click here to download this table.

**Table 7. Proteinogenic amino acids.** This spreadsheet is used to calculate the concentration (in mM) of proteinogenic amino acids from data summarized in **Table 5** and **Table 6**. Means and standard deviations are calculated for further calculations. Please click here to download this table.

**Table 8. Processing of raw data obtained from the quantification of higher alcohols.** Dataset includes data from the measurements of volatile compound concentrations (**A**) and converts data expressed in units of mg/L to µM (**B**). Means and standard deviations are calculated for further calculations. Please click here to download this table.

**Table 9. Processing of raw data obtained from the determination of isotopic enrichments of proteinogenic amino acids using $^{15}$N-labeled substrates**.
Means and standard deviations are calculated for further calculations. Please click here to download this table.

**Table 10. Processing of raw data obtained from the determination of isotopic enrichments of proteinogenic amino acids and volatile compounds using $^{13}$C-labeled substrates**.
Means and standard deviations are calculated for further calculations. Please click here to download this table.

## Discussion

Quantifying the partitioning of compounds through metabolic networks using isotopic tracer experiments is a promising approach for understanding the operation of microbial metabolism. This methodology, while successfully applied with one or two labeled substrates, cannot currently be implemented to study metabolism of various sources using multiple labeled elemental isotopes (*i.e.*, more than two substrates). Indeed, the available analytical techniques enable the accurate determination of the labeling patterns of proteinogenic amino acids and molecules exclusively when using isotopes of a single element and possibly when co-labeling with two elements. Consequently, to address these limitations and assess the management of multiple nutrient sources by microorganisms, we chose to repeat a set of cultures under the same environmental conditions while labeling a selected substrate with $^{13}$C or $^{15}$N isotopes. Then, further combination of the specific information that is provided by each $^{13}$C or $^{15}$N tracer experiment offers a quantitative extended view of the metabolism of multiple sources.

The achievement of the reported approach relies on the implementation of a reproducible series of cultures under standard conditions and on the collection of samples from a population of cells in a defined physiological state that is the same for all cultures (cell growth, consumption of substrate, *etc.*). These conditions must be satisfied so that data obtained from independent experiments can be mixed and analyzed together. Satisfying these constraints requires accurate and frequent monitoring of the microbial activity which was carried out, in the experimental work reported here, using a robot-assisted system for online monitoring of yeast fermentation activity. However, more conventional methods for microorganism cultivation and monitoring, such as the determination of cell growth by measuring the optical density, can be used to normalize the sampling procedure.

Another prerequisite for carrying out this methodology is to have a clear vision of the metabolic pathways that are involved in the network to be investigated. This knowledge is essential for choosing the set of labeled substrates that are the most appropriate for dealing with the issue being studied. The labeled compounds as well as the nature and position of the labeling ($^{13}$C, $^{15}$N, or others) within the molecules must be selected suitably in order to i) recover all the labels provided by the substrates in the compounds that are derived from their catabolism and are further analyzed in the procedure and ii) obtain redundant data that demonstrate the accuracy of the methodology and the validity of the findings. The procedure described here also includes an important number of analyses that could be time-consuming and costly in human and financial resources. Moreover, some analytical constraints can limit the use of this methodology. First, users should ensure that all the analytical methods that are required for the quantification of the compounds produced during microbial metabolism and for the determination of their isotopic enrichment are available. Second, this approach only applies to assessing the metabolism of substrates for which all the conversion compounds are produced in sufficient amounts to be accurately quantified.

In this paper, we applied this workflow to explore the management of multiple nitrogen sources by yeast during wine fermentation. This report offered new insights on yeast metabolism, including the substantial catabolism of most consumed amino acids, combined with their low direct incorporation into proteins, and the major contribution of CCM to supply precursors that are further used for both the *de novo* synthesis of proteinogenic amino acids and the formation of volatile molecules.

More broadly, the approach that is described here can be used for quantifying the partitioning of multiple substrates through the metabolic network of any microorganism. It will allow researchers to elucidate the fate of all consumed compounds after they enter the cells and to address the identification of the metabolic origin of precursors and products. This information can be useful for comparing the metabolic activity of strains that have different genetic backgrounds or grow under different conditions and, consequently, for designing rational strategies to improve fermentation processes.

## Disclosures

The authors have nothing to disclose.

## Acknowledgements

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