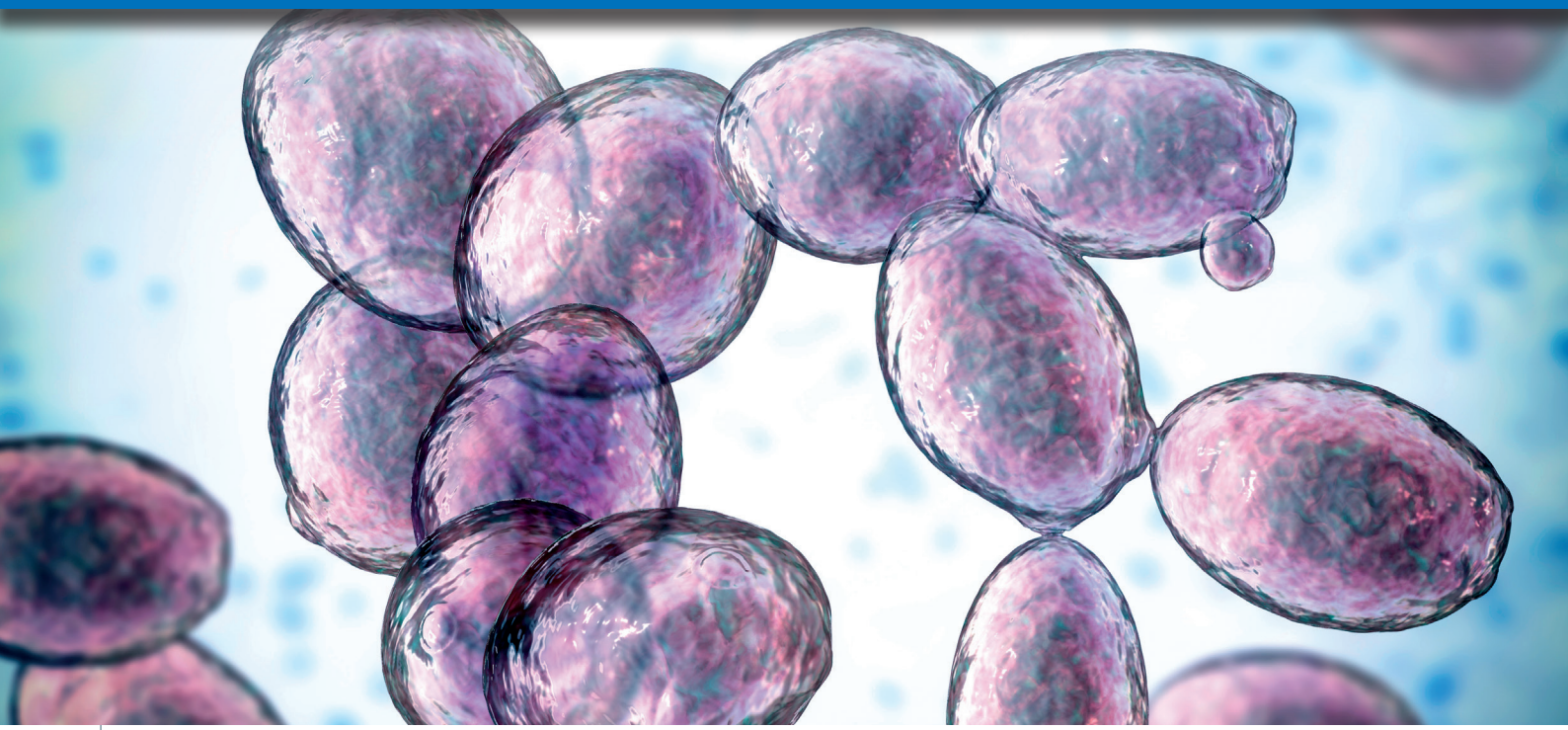

Magnetic Resonance Mass Spectrometry (MRMS) discriminates yeast mutants through metabolomics

An untargeted metabolomics approach based on the MRMS aXelerate® workflow was employed to examine changes in methylglyoxal catabolism using *Saccharomyces cerevisiae* as a model system. This approach allowed for subtle changes in cell metabolism to be revealed and phenotypically identical single-gene deletion mutants of isogenic yeast strains to be accurately distinguished.

Abstract

An eukaryote model of *Saccharomyces cerevisiae* was used to study the methylglyoxal pathway by Magnetic resonance mass spectrometry (MRMS). It was discovered that glutathione plays a major role in driving metabolomic differences between different strains. It is expected that metabolomics-based discrimination of microorganisms surpass other molecular biotyping methods.

Introduction

Saccharomyces cerevisiae is a model eukaryote with around 6000 genes, most of which can be deleted without compromising cell viability. A vast

Keywords: Metabolomics, MRMS, MRMS aXelerate workflow, yeast mutants

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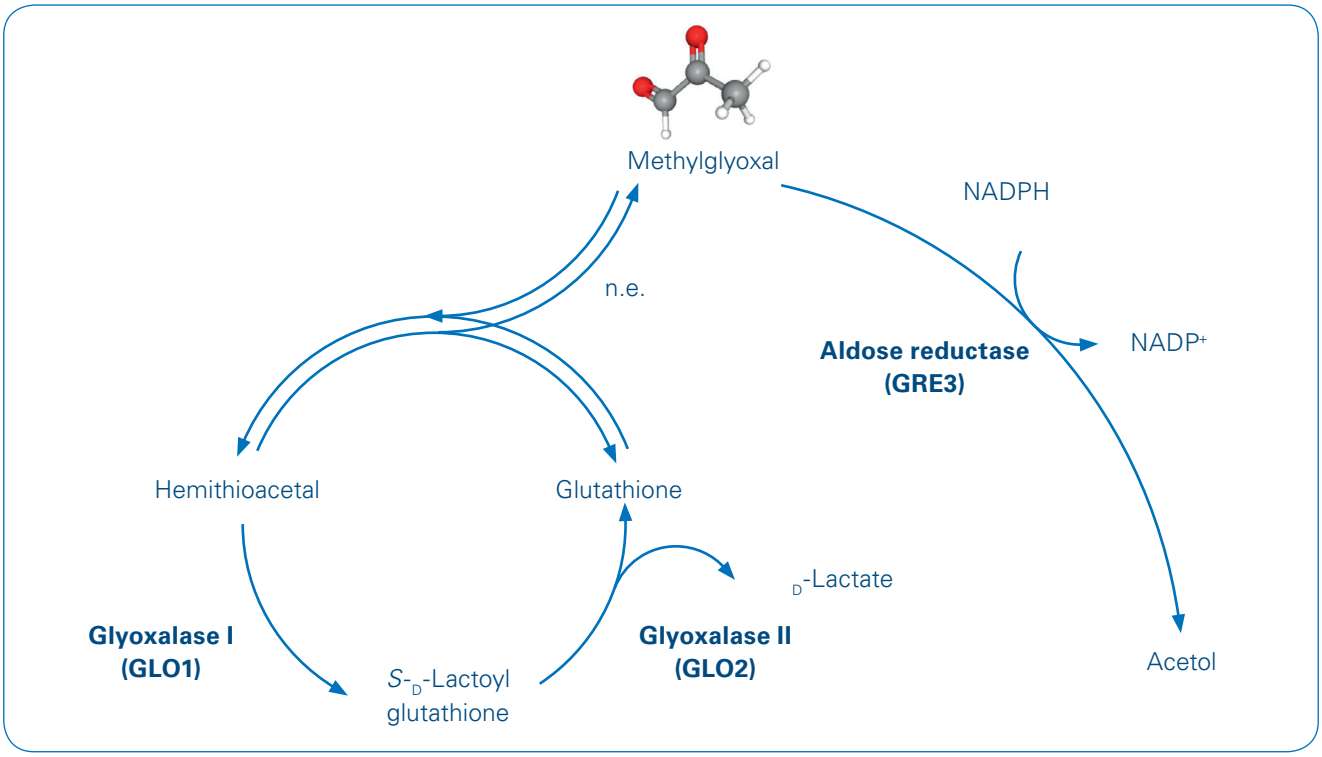

Figure 1. Methylglyoxal metabolism in yeast.

fraction of these mutations is silent, only producing an observable phenotype in specific growth media or under stress conditions (Kuepfer, et al., 2005; Wagner, 2000; Winzeler, et al., 1999). This is the case of yeast mutants related to the methylglyoxal catabolic pathways. Methylglyoxal ($CH_3C(O)CHO$) is a ubiquitous chemical compound found in all cells as an inevitable by-product of metabolism. Mainly formed during glycolysis, it is highly cytotoxic and extremely reactive towards amino groups in proteins and nucleic acids (Sousa Silva, et al., 2013). Cells efficiently eliminate this toxic dicarbonyl (Figure 1), being the glyoxalase system its main catabolic pathway (Sousa Silva, et al., 2013). Through the sequential action of glyoxalase I (GLO1, lactoylglutathione methylglyoxal lyase; EC 4.4.1.5) and glyoxalase II (GLO2, hydroxyacyl-glutathione hydrolase, EC 3.1.2.6), methylglyoxal is converted to D-lactate using glutathione as cofactor. Another methylglyoxal detoxification pathway is the NADPH-dependent reduction to 1,2-propanediol, a two-step reaction catalyzed by aldose reductase (GRE3, aldehyde reductase, EC 1.1.1.21), (Vander Jagt & Hunsaker, 2003). Despite the increased concentration of intracellular methylglyoxal and the detection of glycated proteins in these three yeast mutants, they are all viable and do not present any growth impairment, since cells efficiently modulate the two catabolic systems to cope with methylglyoxal (Gomes, et al., 2005).

Discriminating these strains would hardly be achievable through full genome sequencing, because they differ only by one gene, or through proteomics, where just a single protein would be missing. Could subtle changes in methylglyoxal catabolism be revealed through an untargeted metabolomics approach based on extreme mass resolution and mass accuracy using Magnetic Resonance Mass Spectrometry (MRMS)?

Sample preparation and analysis

Yeast strains and growth

Saccharomyces cerevisiae strains from the Euroscarf collection (Frankfurt, Germany) included the reference strain BY4741 (genotype: MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0) and 3 isogenic single-gene deletion mutants: ΔGLO1, ΔGLO2, and ΔGRE3. All yeast strains were grown in YPD medium at 30°C for 14 h, until the end of the exponential growth phase.

Metabolite extraction and MRMS analysis

Metabolites were extracted from a pellet of 2 mL grown cells by resuspending them in 1 mL of methanol (LC-MS grade)/water (1:1), followed by three cycles of 1 min vortex / 1 min incubation on ice. The supernatant was recovered after

centrifugation, diluted 1:100 in methanol/water (1:1) and 0.1% formic acid was added to each sample. Human leucine-enkephalin was added as internal standard for online lock mass calibration ($[M+H]^+$ = 556.27657 Da).

Samples were analyzed by direct infusion using a solariX XR MRMS (Bruker Daltonics GmbH & Co. KG, Bremen, Germany) equipped with a 7 T superconducting magnet. Samples were analyzed in positive electrospray ionization mode (ESI+). Three replicates were collected for each yeast strain. Mass spectra were acquired in magnitude mode, in the mass range between 200 and 1200 *m/z*, with a 4 M transient resulting in a mass resolution of 1,000,000 @ *m/z* 400. Hundred single scans were added for the final mass spectrum.

Data analysis

Raw data was analyzed by MetaboScape® 4.0 (Bruker Daltonics GmbH & Co. KG, Bremen, Germany) using the T-ReX® 2D algorithm. All samples' peak lists were aligned in a single bucket table and the intensities were normalized with the internal standard (leucine enkephalin). Possible molecular formulas for each mass were determined using the SmartFormula function (0.2 ppm maximum mass deviation) and putative annotation of metabolites performed using the analyte lists from yeast (YMDB, Ramirez-Gaona, et al., 2017) and the Human (HMDB, Wishart, et al., 2007) Metabolome Databases, uploaded to MetaboScape (maximum mass deviation of 0.2 ppm).

Multivariate statistical analysis was performed in MetaboScape. Principal Component Analysis (PCA) models were built by applying Pareto scaling, retaining a minimum number of principal components necessary to explain 98% of variance. Sample Hierarchical Clustering (agglomerative) was performed considering an Euclidean distance and using the Ward distances method. To identify the compounds that better discriminate between yeast samples, a Partial Least Squares Discriminant Analysis (PLS-DA) was performed, defining two different groups: strains with mutations in the glyoxalase enzymes (ΔGLO1 and ΔGLO2) and strains with both glyoxalases functional (BY4741 and ΔGRE3). Variable Influence on Projection (VIP) scores were calculated and the specific metabolites that contributed the most to the differentiation between the strains were identified.

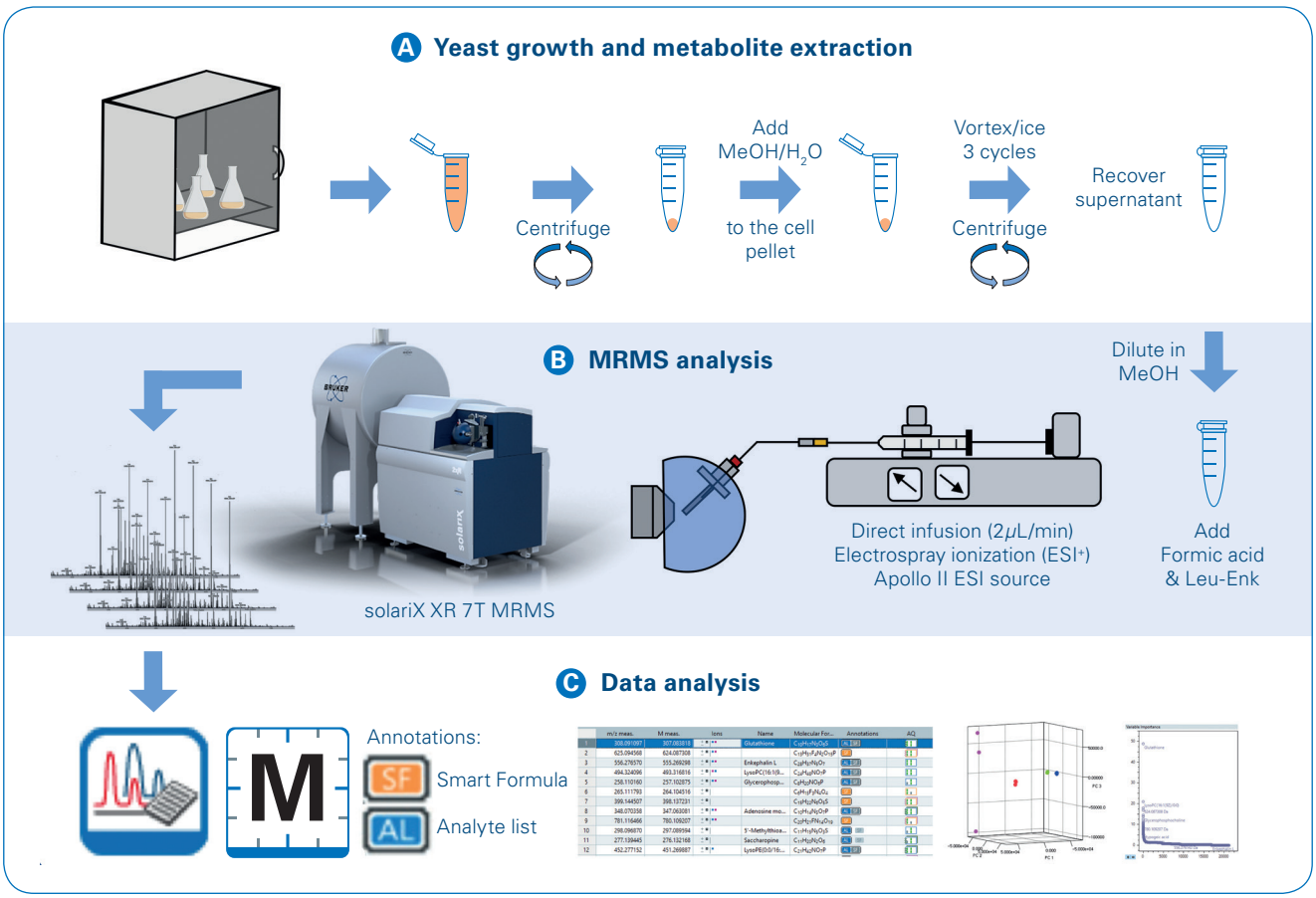

Figure 2. Schematic of the MRMS aXelerate workflow including (A) yeast growth and metabolite extraction, (B) MRMS analysis and (C) data analysis.

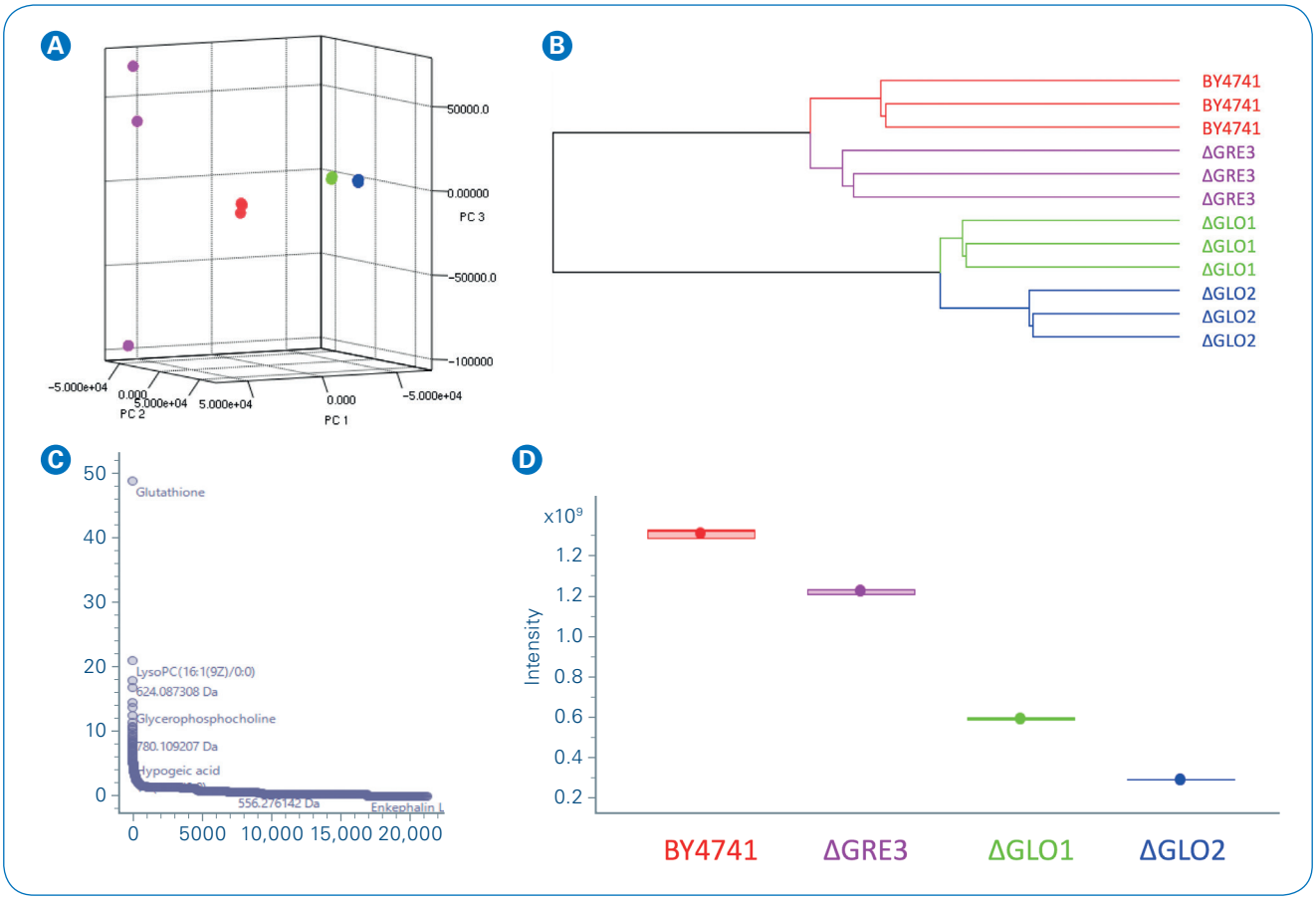

Figure 3. Multivariate statistical analysis applied to the metabolomics data of the yeast samples. (A) Principal components analysis using the first three principal components; (B) Hierarchical clustering analysis; (C) Variable importance (VIP), calculated from PLS-DA analysis; (D) Bucket statistic (box plots) of glutathione in different yeast samples.

Results

An untargeted metabolomics analysis using direct infusion MRMS was performed to analyze the chemical profiles in all yeast strains. After spectra alignment in MetaboScape, a total number of 21,174 features were obtained. The number of identified assigned features using the HMDB database were 624. Using SmartFormula 3943 features were assigned based on molecular formula. Principal component analysis (PCA) and hierarchical clustering analysis (HCA) were applied to validate data reproducibility and to detect inter-group metabolic similarities among the various yeast strains. A clear separation of all strains was observed in the PCA score plots, with low variability between replicates from the same sample (Figure 3A). Hierarchical clustering also confirms this separation, further supporting the high reproducibility of the method (Figure 3B). This separation trend suggests that multivariate statistical analysis of the yeast samples metabolic profiles, obtained by MRMS, can discriminate between all strains, that belong to the same species and are isogenic. Interestingly, samples show clear separation into two groups: one with the reference BY4741 and ΔGRE3 strains, and the other with both mutants for the glyoxalase pathway enzymes, ΔGLO1 and ΔGLO2.

To identify the metabolites that contributed the most for this separation, a Partial Least Squares Discriminant Analysis (PLS-DA) model was fitted to the MS intensity data, building a system of components that maximized covariance between the groups (in this case, belonging and not belonging to the glyoxalase pathway). Glutathione showed the highest VIP score (Figure 3C, D), contributing to the separation, not only between the 2 defined groups, but also between the 4 strains. Glutathione was identified using the HMBD and YMDB databases, and by molecular formula assignment detected as protonated species ($[M+H]^+$ = 308.09109 Da). Moreover, extreme mass resolution,

mass accuracy and dynamic range achieved by MRMS during the analysis of this very complex mixture, allowed the identification of 12 of its isotopologues thus establishing its molecular formula unequivocally (Figure 4).

Glutathione is a key metabolite in the methylglyoxal catabolism, particularly related to the glyoxalase pathway. In this study, decreased levels of glutathione were observed in the ΔGRE3 strain, where oxidative stress may be at play. The largest decrease was found in the ΔGLO2 strain that is unable to regenerate reduced glutathione. Other compounds belonging to the glutathione metabolic pathway were also identified, although their relative concentration was not the same in all strains. All the detected metabolites in this pathway were more abundant in the reference strain (BY4741), meaning that the single-gene mutations analyzed caused these changes in the metabolism, among others.

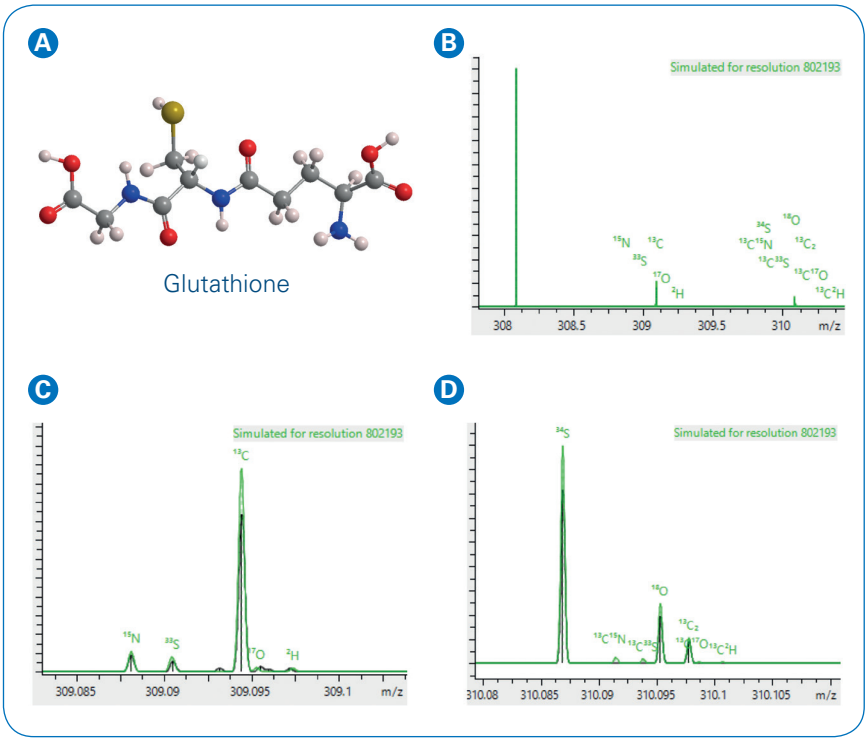

Figure 4. Glutathione fine isotopic distribution. (A) Glutathione structure. (B) Experimental and simulated glutathione fine isotopic distribution (for mass resolution of 800k), highlighting the (C) second and (D) third isotopes. Black, experimental; green, theoretical isotopic distribution.

Conclusion

- An untargeted metabolomics approach based on the MRMS platform providing extreme mass resolution can accurately distinguish between phenotypically identical single-gene deletion mutants of isogenic yeast strains.
- A clear separation into two groups, one comprising the ΔGLO1 and ΔGLO2 mutant strains, and the other with ΔGRE3 and BY4741, was achieved.
- Glutathione plays a central role in driving the metabolic differences between the strains, being its relative abundances responsible for the 2 groups' separation, since glutathione regeneration is impaired in ΔGLO1 and ΔGLO2.
- Phenotypically identical single-gene deletion mutants of the same yeast were separated, revealing not only a vast array of metabolic differences, but also some unexpected similarities between them.
- We expect that metabolomics-based discrimination of microorganisms will surpass other molecular biotyping methods, given the wealth of information provided that supports a greater discrimination power.

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