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Monitoring Alcoholic Fermentation: An Untargeted Approach

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ABSTRACT: This work describes the utility and efficiency of a metabolic profiling pipeline that relies on an unsupervised and untargeted approach applied to a HS-SPME/GC-MS data. This noninvasive and high throughput methodology enables "real time" monitoring of the metabolic changes inherent to the biochemical dynamics of a perturbed complex biological system and the extraction of molecular candidates that are latter validated on its biochemical context. To evaluate the efficiency of the pipeline five different fermentations, carried on a synthetic media and whose perturbation was the nitrogen source, were performed in 5 and 500 mL. The smaller volume fermentations were monitored online by HS-SPME/GC-MS, allowing to obtain metabolic profiles and molecular candidates time expression. Nontarget analysis was applied using MS data in two ways: (i) one dimension (1D), where the total ion chromatogram per sample was used, (ii) two dimensions (2D), where the integrity time vs m/z per sample was used. Results indicate that the 2D procedure captured the relevant information more efficiently than the 1D. It was also seen that although there were differences in the fermentation performance in different scales, the metabolic pathways responsible for production of metabolites that impact the quality of the volatile fraction was unaffected, so the proposed pipeline is suitable for the study of different fermentation systems that can undergo subsequent sensory validation on a larger scale.

KEYWORDS: *metabolomic, fermentation, target, untargeted, HS-SPME/GC-MS, PCA, OPLS-DA*

INTRODUCTION

The monitoring of the fermentation process is a common procedure in wine research that often requires large samples, expensive equipment, and skilled labor. Model fermentations are generally carried out under controlled conditions when studying yeast metabolism and the accompanying metabolome. These fermentations are generally carried out on volumes between 80 and 500 mL and are mostly only analyzed at the end of fermentation.[1−3] The monitoring of the fermentation process is usually based on optical density and weight loss measurements as indicators of yeast growth and performance. Additionally, these fermentations would require at least 3 biological replicates in order to reliably infer meaning from the data obtained, which reduces the number of perturbations evaluated.

One of the main objectives of technological research is the development of equipment and techniques capable of performing a great number of experiments in the smallest volume, that is, in high throughput.

The fermentation process is affected by many factors such as yeast strain, temperature, oxygen, nutrients, among others.[4−12] The metabolic effect of these factors on a fermentation have a major impact on the final product quality and consequently on its comercial value. The development of a protocol, requiring only pre-existing equipment, capable of following the online changes in the volatile fraction may enable the construction of metabolic networks characteristic of each of the different fermentation systems. This would be of great interest to aroma research, as well as to the wine industry.

Many researchers have highlighted the importance of the nitrogen content of a must, in terms of both the nitrogen composition and the total nitrogen available.[8−10,12−15] The nitrogen content directly impacts the final aroma composition in terms of the production of higher alcohols, volatile fatty acids, esters, and sulfur and carbonyls compounds.[14,15] However, this effect is highly complex and the link between a specific nitrogen composition and the aromatic profile is not well understood.[4,13]

Metabolomics studies metabolites and their chemical features in a biological system in order to identify or discover new compounds and elucidate the effects their presence has on biochemical pathways (either known or unknown) leading to a better understanding of cellular behavior.[16−19] Metabolites are a group of low molecular weight substances (50−1500 Da) that includes amino acids, fatty acids, lipids, purines, pyrimidines, carbohydrates, peptides, hormones, volatile metabolites, and many more organic molecules serving as intermediates/substrates and products in cellular reactions (the metabolic pathways). The ultimate aim of metabolomics is to obtain a so-called metabolic fingerprint leading to an understanding of the entire metabolic network. To achieve a holistic view of metabolites in their biochemical context it is necessary to identify known metabolites under analytical conditions in the data set. However, in most instances, targeted chemical analysis

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is performed, and due to the complexity of the yeast metabolome, there is a loss of information because the focus is not on the overall system. An untargeted approach allows the visualization of the process and reveals new insights by detecting as many metabolites as possible in a single step.[17,19] In an attempt to get as close as possible to this holistic metabolic view of a complex biological process, the aim of the present work is to build a metabolic profiling pipeline able to identify metabolites from a perturbed data set and latter validate them in their biochemical context. This newly developed methodology reproduces in a micro scale (5 mL) the same metabolic events that are usually monitored in a larger scale enabling the high throughput analysis of samples. The 5 mL fermentations were monitored online and noninvasively through the continuous sampling of the volatile fraction by headspace—solid phase microextraction (HS-SPME) analyzed by gas chromatography coupled to a mass spectrometry detection system (GC-MS) in order to capture as much information regarding the fermentation process as possible.

In order to evaluate the proposed pipeline a commercial stain of *Saccharomyces cerevisiae* fermented synthetic grape must containing the same concentration (200 mg/L) of different sources (ammonium and amino acids) of yeast assimilable nitrogen. These 5 mL fermentations were analyzed and the pipeline was validated by comparing them to a second set of fermentations performed on a larger scale (500 mL). The metabolic information was subjected to preprocessing through algebraic normalization (Pareto) using MarkerLynx, both in one dimension (1D), where the total ion chromatogram per sample was used and in two dimensions (2D), where the integrity time vs m/z per sample was used. Multivariate variable analysis (MVA) was used to evaluate the potential of a high throughput pipeline. This type of approach needs validation with the study of real time perturbations in a time course of some chemical compounds (targeted approach).

MATERIALS AND METHODS

Chemicals. The highest purity chemicals were used throughout the experiments. All chemicals were from Sigma-Aldrich (Germany) or Merck (Germany) unless otherwise stated.

Strain, Media, and Culture Conditions. The yeast strain used in this study was *Saccharomyces cerevisiae* VIN13 (Anchor yeast, Cape Town, South Africa). The yeast preculture was grown in the fermentation medium described below and containing NH_4Cl (1g/L) as nitrogen source. The yeast, precultured to the logarithmic growth phase, was centrifuged and resuspended in saline solution. Fermentation vessels were inoculated with the preculture to an OD_{600} of 0.1 (final cell density of approximately 10^6 cells/mL).

Fermentations were carried out in grape juice medium adapted from Jiranek et al.[20] The synthetic media was prepared by the combination of two aqueous solutions prepared and sterilized separately. The first solution contained glucose (100 g/L), fructose (100 g/L), citric acid (0.2 g/L), malic acid (3 g/L), K_2HPO_4 (1.14 g/L), $MgSO_4{\cdot}7H_2O$ (1.23 g/L), $CaCl_2{\cdot}2H_2O$ (0.44 g/L), KH tartrate (2.5 g/L). All the compounds were dissolved in distilled water, and the pH was set to 3.3 with KOH 10 M. This solution was autoclaved for sterilization. The second solution contained NH_4Cl and/or amino acids as described in Table 1, trace elements stock (final concentration in the synthetic grape must: $MnCl_2{\cdot}4H_2O$ 200 μg/L, $ZnCl_2$ 135 μg/L, $FeSO_4$ 36 μg/L, $CuCl_2$ 15 μg/L, H_3BO_3 5 μg/L, $Co(NO_3)_2.6H_2O$ 30 μg/L, $NaMoO_4.2H_2O$ 25 μg/L, and KIO_3 10 μ/L) and vitamin stock (final concentration in the synthetic grape must: myo-inositol 100 mg/L, pyridoxine HCl 2 mg/L, nicotinic acid 2 mg/L, Ca pantothenate 1 mg/L, thiamine HCl 0.5 mg/L, K para-aminobenzoate 0.2 mg/L, riboflavin 0.2 mg/L, biotin 0.125 mg/L, and folic acid 0.2 mg/L).[20]

Table 1. Different Fermentation Media (M1−5)

media	description
M1	without amino acids just 200 mg N/L of NH_4Cl (50 mg/L) (control)
M2	NH_4Cl (50 mg/L) and cysteine, alanine, arginine, asparagine, aspartic acid, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine (150 mg/L)
M3	NH_4Cl (50 mg/L) and the preferred amino acids (arginine, asparagine, aspartic acid, glutamine, glutamic acid) (150 mg/L)
M4	NH_4Cl (50 mg/L) and the amino acids precursors of "aroma compounds" (isoleucine, leucine, phenylalanine, tyrosine, valine) (150 mg/L)
M5	NH_4Cl (50 mg/L) and S-containing amino acids (cysteine and methionine) (150 mg/L)

This second solution was filter sterilized and added to the first solution.

Experimental Data Set. The synthetic grape must for each treatment (M1−5) contained 200 mg/L of a nitrogen source which varied in its composition (Table 1). For the evaluation of the scale effect each fermentation treatment was performed in two volumes, 5 and 500 mL. Each 5 mL sample (M1−5 × 3 replicates) was sampled and analyzed directly after one another nine times (intervals of almost 45 min). The 500 mL samples (M1−5 × 3 replicates) were sampled daily for volatile fraction analysis.

In order to monitor and synchronize all the fermentations, two additional sets of the 5 mL fermentations were carried out. One set was frequently sampled for optical density (OD) measurements at 600 nm. The second set of the 5 mL fermentation replicates was used only for weight measurements. The 500 mL fermentations were monitored frequently for both OD and weight measurements. For logistical reasons, the 5 mL fermentations were only monitored for the first 150 h after inoculation. The 500 mL were monitored to the end in order to evaluate whether the yeast was able to complete the fermentation process under the conditions tested.

Growth Measurement. Cell proliferation was determined with a spectrophotometer (Powerwave$_x$, Bio-Tek Instruments, Bedfordshire, U.K.) by measuring the optical density (600 nm) over the experimental period.

Volatile Analysis. A small-scale fermentation setup (5 mL) was designed using the 15 mL SPME vials for the CombiPAL auto sampler (CTC Analytics AG, Zwingen, Switzerland). Fermentations were carried out on the 32-sample tray and were left to ferment at ambient temperature (20 °C ± 2). In order to avoid the influence of any additional stress, such as heating and agitation, sampling took place directly out of the tray. A SPME (CAR/DVB Carboxen) fiber (Supelco, Bellefonte, PA) was used to collect headspace volatiles every 24 h from each sample. Elevated pressure due to CO_2 production was avoided by inserting a small piece of fused silica GC column in the septum of the vial-cap.

An automated system for the online process monitoring was used thus allowing the monitoring of the fermentation evolution over time. The 5 mL samples taken daily from the 500 mL fermentation were placed in identical vials and analyzed through the same procedure as the 5 mL fermentations. Sampling took place from the headspace of the fermentation vials with an extraction time of 10 min at ambient temperature (20 ± 2 °C) directly from the sample tray. Afterward the SPME fiber was thermally desorbed in the injection port of the GC-MS instrument (Agilent Technologies, Little Falls, Wilmington, U.S.A.) for 1 min at 240 °C in splitless mode and left for a further 9 min in the split mode to clean the fiber. The carrier gas used was helium, the split flow was 50 mL·min^{-1}, and the flow through the column was 1.2 mL·min^{-1}. The column used was a J&W FFAP (Free Fatty Acid phase, Agilent Technologies) of 60 m length, 0.25 mm inner diameter, with a film thickness of 0.5 um. The GC oven temperature program was as follows: 40 °C held for 2 min and then ramped to 240 °C at 10 °C·min^{-1} and held for 2 min. The transferline to the MS was heated to 250 °C, and the ion source temperature and the quadrupole temperature was set to 240 and 150 °C, respectively.

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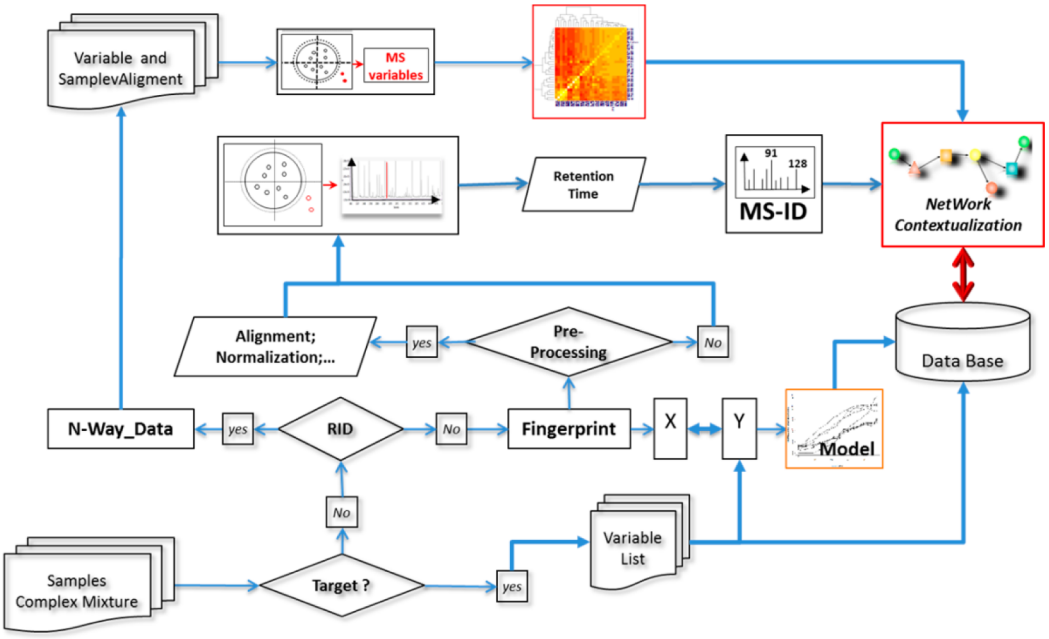

Figure 1. Metabolomic analysis pipeline.

The MS was operated in full-scan mode, scanning from 35 to 650 amu, with and ionization energy of 70 eV and an electron multiplier voltage of 1541 V.

Data Preprocessing. The ASCII file of chromatographic data obtained from each sample was extracted and a matrix created containing all the Total Ion Count features (TIC-Fingerprint). The normalized matrix was then imported into The UnscramblerX 10.1 (Camo, Norway) where baseline correction was performed and the first stage of the alignment of chromatograms done using correlation optimized warping. This algorithm aligns chromatograms by means of sectional linear stretching and compression, which shifts the peaks of one chromatogram to correlate with those of the other chromatograms in the data set.[21]

MarkerLynx XS (version 4.1, Waters, U.S.A.) was also used for peak deconvolution, peak integration (using apex track peak detection), and sample alignment. The peaks from different samples were aligned so that the same peaks (defined with retention time and m/z), most probably the same compound, are placed in the same row for all samples. The parameters used were XIC window 0.50 Da, peak width at 5% height 20 s, intensity threshold 1000 counts, mass window 0.45 Da, retention time window 0.10 min and noise elimination level 10. The list of features originated was then subjected to multivariate analysis. The matrix resulted from MarkerLynx underwent correlational analysis using Excel.

Normalization Methods. The fingerprint data (TIC) and the matrix generated in MarkerLynx were subjected to normalization: mean centering and Pareto normalization (dividing each feature by the square root of the standard deviation).

Statistical Analysis. The relative growth rates and CO_2 liberation in different volumes and different media were evaluated via two-way analysis of variance (ANOVA), using Microsoft Office Excel, version 2010.

Data was analyzed with PCA (principal component analysis), and OPLS-DA, using SIMCA-P+ (version 12.0.1, Umetrics, Sweden). Due to the large number of variables and few observations these methods highlight important information by correlating the variables.

PCA is an unsupervised technique that classifies samples according to their common spectral characteristics facilitating the observation of relationships between samples and highlighting the variables responsible for the variation (it projects the total variation on a plane, onto a smaller set of variables, called principal components (PCs), which are a linear combination of all the initial variables). The scores (the original data in the new system, a projection of the samples) and loadings (the weights applied, the original variables) plots may reveal clusters or outliers and the corresponding variables that have influence on the distribution of samples.

OPLS-DA is a modified version of PLS (partial least-squares), a supervised method, allowing the extraction of more information used for group classification/separation. The advantage is that the information about the discriminating classes is presented in its first component which makes easier to understand which variables are the responsible for this same separation due to the separation of systematic variation into a predictive and an orthogonal compound.[22] The sequence presented in this section is the metabolomics pipeline applied as represented in Figure 1.

RESULTS/DISCUSSION

Fermentation Kinetic Parameters. For logistical reasons, the 5 mL fermentations were only monitored for the first 150 h of the fermentation (Figure 2c, g). The 500 mL fermentations were monitored to the end in order to evaluate whether the yeast was able to complete the fermentation process under the nutrient conditions tested (Figure 2a, e). The percent weight loss (CO_2 production) was measured in order to evaluate the fermentation kinetics in both fermentation sets (Figure 2e, g). The relative rates of CO_2 production were calculated using the slope of CO_2 production vs time (Figure 2h). For the 500 mL fermentation, the slope was calculated for the 150 h period (Figure 2f). When the monitoring of the 5 mL fermentations cessed, they had lost 43% of the total CO_2 released by the 500 mL fermentations. Therefore, only this period is the focus of this study. A two-way analysis of variance (ANOVA) was applied to the relative rates of CO_2 production and the data showed that there were no significant differences between different media or different volumes ($p > 0.05$).

The yeast growth was also monitored for the entire fermentation process in 500 mL and until yeasts reached a quasi-stationary phase (OD of about 6) in 5 mL fermentations. The relative rates of cell growth were calculated using the slope of OD (600 nm) vs time in the exponential growth phase (Figure 2d). Once again for the 500 mL fermentations, only the data for the first 150 h was considered (Figure 2b). A two-way

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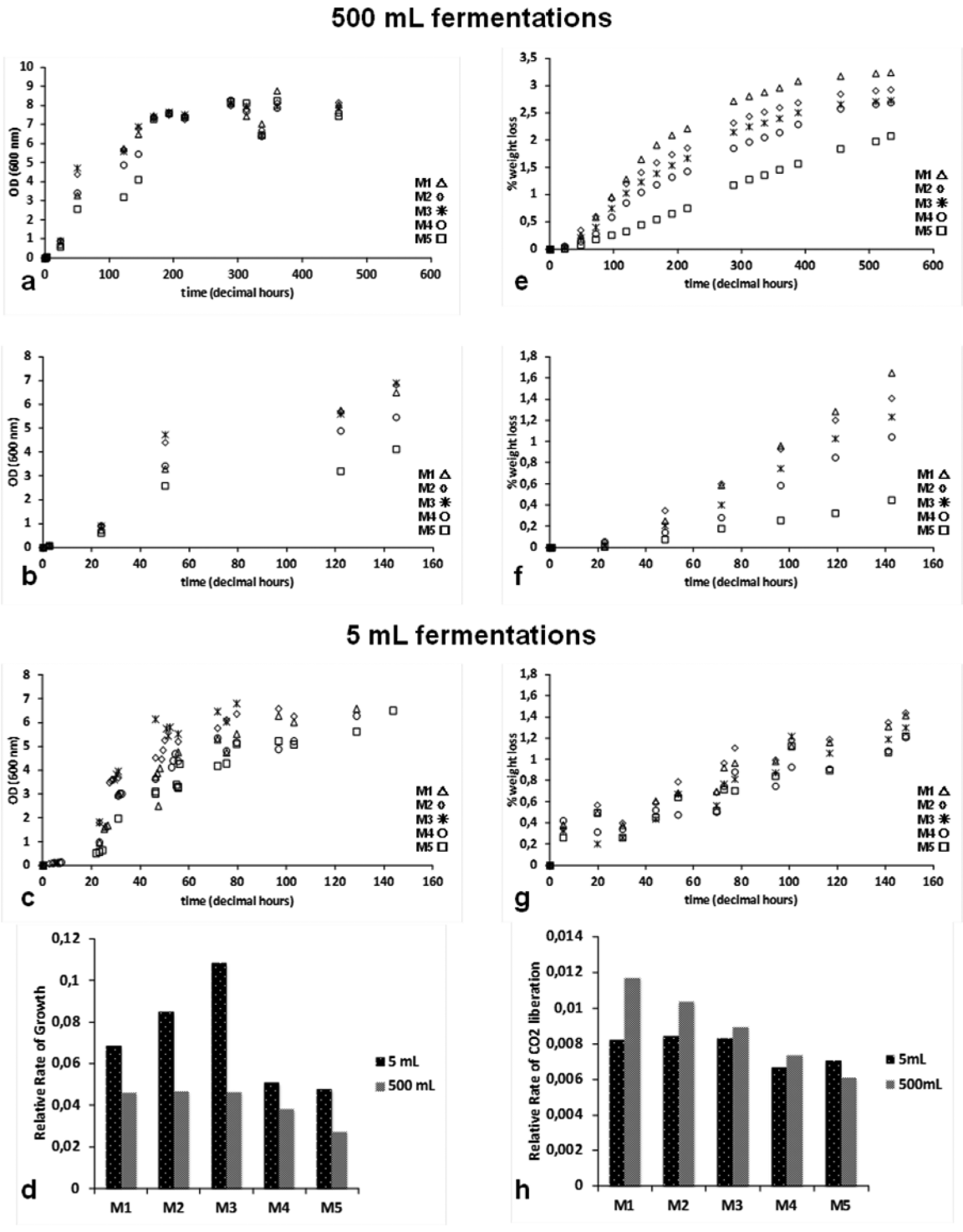

Figure 2. Profiles of yeast growth obtained through OD measurements at 600 nm in (a) 500 mL fermentations for the entire fermentation process and (b) for the first 150 h, (c) in 5 mL fermentations for 150 h and (d) relative rates of growth; % weight loss (CO_2 production) in 500 mL fermentations (e) for the entire fermentation process and (f) for the first 150 h (g) in 5 mL fermentations for 150 h and (h) relative rates of CO_2 production.

analysis of variance (ANOVA) was applied to this data, and it was concluded that although there were no significant differences between media ($p > 0.05$) there were significant differences between volumes ($p < 0.05$).

Data from the entire 500 mL fermentations process (Figure 2a, e) showed that despite the differences in the nitrogen composition of different media, *Saccharomyces cerevisiae* was able to perform the fermentation process in all media. In a media completely deficient in amino acids the yeast has the ability to use precursor metabolites, carbon skeletons originated from the central metabolic pathways, as starting substrates for the synthesis of amino acids. The tricarboxylic acid cycle is not functional during fermentation, so yeasts replace the cycle intermediates through anaplerotic reactions.[23,24]

Screening by TIC. Our initial goal with the fingerprint analysis (TIC) was to examine the intrinsic variation in both

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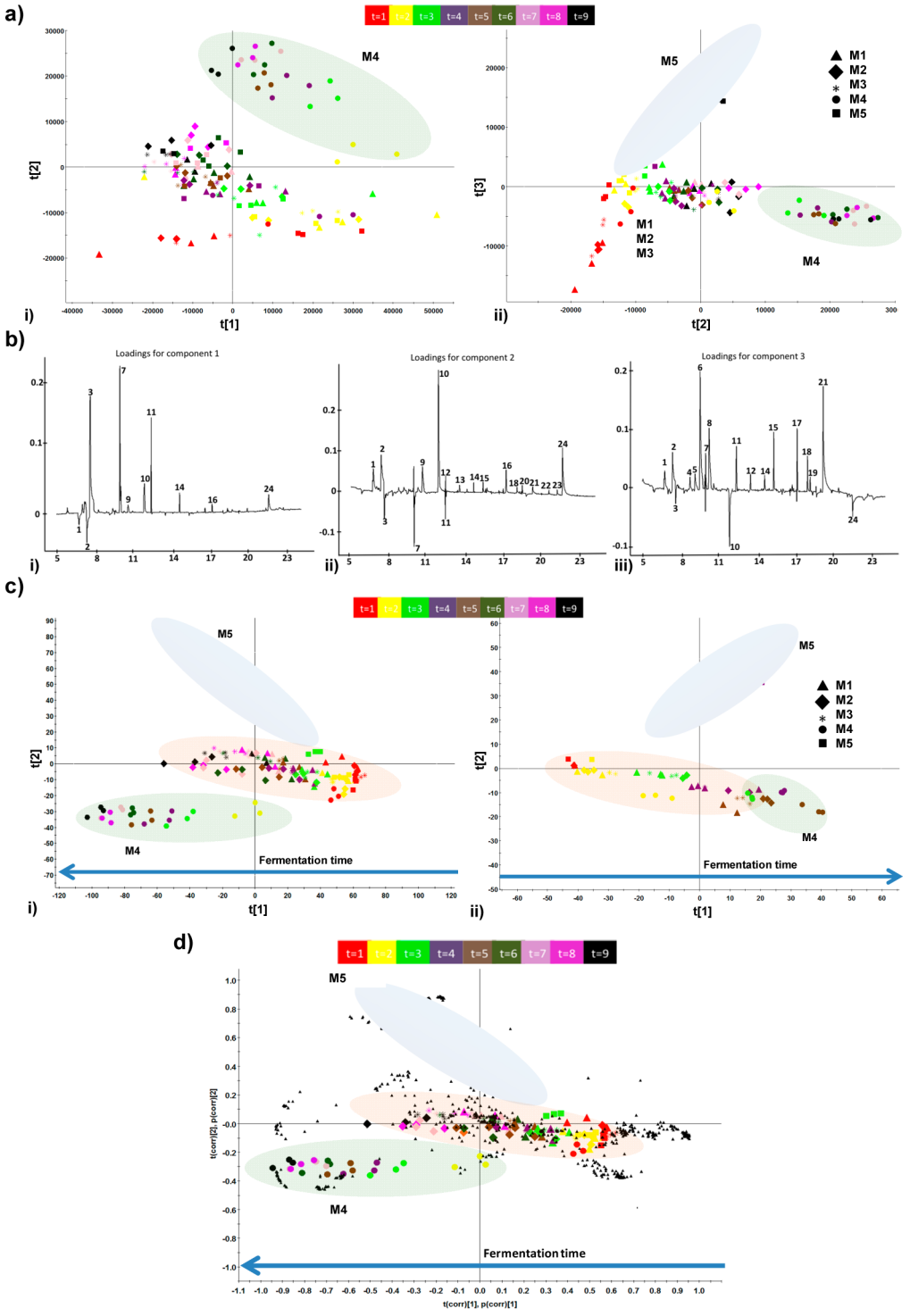

Figure 3. (a) PCA score plots of normalized raw data. (i) PC1 vs PC2; (ii) PC2 vs PC3. (b) PCA loading plots of normalized raw data for (i) PC1, (ii) PC2, (iii) PC3 for (1) ethyl acetate; (2) ethanol; (3) unknown 1; (4) isobutyl acetate; (5) ethyl butanoate; (6) methyl thiolacetate; (7) 2-methyl-1-propanol; (8) ethyl thiolacetate; (9) isoamyl acetate; (10) 3-methyl-1-butanol; (11) unknown 3; (12) ethyl hexanoate; (13) acetoin; (14) unknown 4; (15) ethyl octanoate; (16) benzaldehyde; (17) dihydro-2-methyl-3(2H)-thiophenone; (18) ethyl decanoate; (19) 3-methylsulfanylprop-1-ene; (20) unknown (74//60/41); (21) methionol; (22) phenylethyl acetate; (23) benzyl alcohol; (24) 2-phenylethanol. (c) PCA score plots of normalized data after preprocessing with MarkerLynx and normalization by Pareto (i) 5 mL, (ii) 500 mL. (d) Biplot of the two first PC after preprocessing with MarkerLynx and normalized by Pareto (5 mL fermentations).

data sets to determine if the volatile fraction of the samples followed similar trends and/or media clustering prior to feature extraction techniques.

The raw data was first evaluated by PCA using the non-normalized data (data not shown) to verify the samples, and to detect outliers or trends within the different media applied in the TIC-Fingerprint matrix. For the 5 mL data set, the first three principal components showed 97% of the total variation and it was possible to observe a cluster with samples related to M4 (branch chain and aromatic amino acids). However, it was

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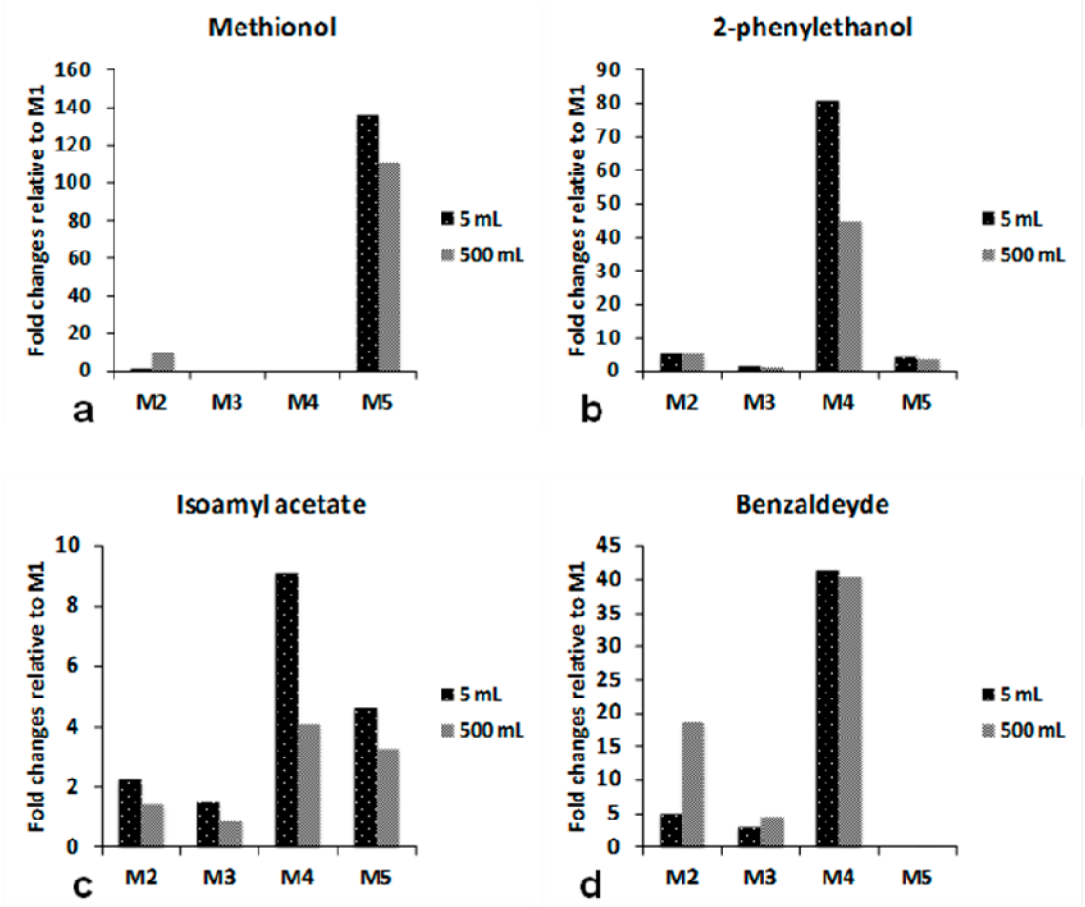

Figure 4. Fold changes relative to M1 (control) in 5 and 500 mL fermentations for (a) methionol, (b) 2- phenylethanol, (c) isoamyl acetate, and (d) benzaldehyde.

necessary to reach component 6 to visualize another cluster with the data of M5 (sulfur-containing amino acids) (just 0.37% of the variation was explained by this component). In the case of the 500 mL data set, the first three principal components showed 95% of the total variation, with M4 forming a cluster. To see another cluster it was necessary to reach component 4 (2% of variation explained). The second cluster found was for M5 as seen with the 5 mL fermentations. This suggests that metabolites, such as the volatile compounds produced in M4, may dominate and in so doing obscure others (such as the volatile compounds from S-containing amino acids medium). This may be overcome performing a mathematical normalization on data to minimize these differences. Pareto normalization is used to avoid problems such as baseline influence and variations in peak shapes, among others; the raw data was compared to this data set. The results confirmed that some metabolites hide others. The plots (Figure 3a, b) show that the second component separates the data from M4 and the third explains the medium containing sulfur amino acids (M5), while the first component is explained by fermentation products (with 79% and 88% (data not shown) of the total variation for the 5 and 500 mL samples, respectively).

2D "Landscape" Data Preprocessing. *Effect of Sample Discrimination.* In PCA, each chromatogram and all its information is compressed and projected onto a single point. Each point represents the entire exogenous volatile metabolome at a moment. PCA was performed to understand how samples cluster together (scores) (Figure 3c). These PCA score plots show that the metabolism of yeast is affected by the amino acids supplied in the media but not by the volume of media. In the case of the 5 mL data set, three principal components explained 67% of the total variation while in the 500 mL data set this explained 44%. For both fermentation volumes, the first component (x vector) describes the fermentations over time (kinetics). Indeed, at the beginning of fermentation (initial sampling point) there were no differences in the volatile metabolome, suggesting that the yeast is initiating metabolic pathways in response to the different fermentation media. In the second component, orthogonal to the first one, the result was, once again, similar for both 5 and 500 mL samples. It was possible to distinguish three clusters: M5 samples (cysteine and methionine), M4 samples (phenylalanine, tyrosine, valine, leucine, and isoleucine), and a cluster consisting of M1, M2, and M3. This suggests that it is possible to track the different metabolic pathways that yeast follows over time due to nutrient variation. When evaluating each medium (M) separately, a time dependent expression pattern emerged. For example, in the 5 mL fermentation score plot (Figure 3c(i)), medium M4 displays a time separation between sampling points 3, 4, and 5 whereas the last ones (6, 7, 8, and 9) are grouped in a shorter time range. These differences in volatile compound synthesis are potentially related to variations in gene expression between yeast subjected to different nutrient conditions. Differences in gene expression and consequent proteomic and metabolomic changes enable yeast to survive regardless the nutrient availability.[23,24]

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A biplot was constructed in order to understand and analyze how samples cluster together (scores) and identify which variables (metabolites) most contributed to their separation (loadings) (Figure 3d). This potentially allows metabolic discovery through the identification of possible biomarkers.[25] The biomarkers identified in each media were the same in both 5 and 500 mL samples. It was possible to identify ethyl acetate and ethanol for the cluster formed with M1, M2, and M3. Ethanol is one of main products of fermentation and ethyl acetate is formed from ethanol and acetyl-CoA by alcohol acyltransferase and is the most abundant ester produced during alcoholic fermentation.[13,26] This cluster contains media that placed fewer nutrient constraints on yeast growth as they contained glutamate and ammonia, which are the principal precursors for the synthesis of nitrogenous compounds.[24] This may somewhat contribute to the similarities in the volatile profiles detected. The compounds identified as being responsible for the clustering of M5 were sulfur containing compounds which originated from cysteine and methionine metabolism, such as methyl thioacetate, ethyl thiolacetate, dihydro-2-methyl-3(2H)-thiophenone, and methionol (a product of methionine degradation via Ehrlich pathway[27]) (Figure 4a). These may have a negative contribution to aroma due to the formation of thiols and sulfides.[28]

The clustering of M4 was due to the presence of some products resulting from the Ehrlich pathway, namely isoamyl alcohol and 2-phenylethanol (Figure 4b) together with hexanoic acid and benzyl alcohol. The higher alcohols isoamyl alcohol and 2-phenylethanol are formed by the decarboxylation and subsequent reduction of α-keto acids produced as intermediates of the amino acids leucine, isoleucine, and phenylalanine catabolism (Ehrlich pathway). Since their production from the corresponding amino acids is coupled to the oxidation of NADH, in anaerobic conditions yeasts use them as alternative electron acceptors.[29] These results are in agreement with literature.[30−32]

In order to gain more information and to contextualize each pathway in the global yeast metabolome an OPLS-DA between media was applied. The results generated with this approach corroborate the presence of the compounds identified in the PCA plots. Additionally, it was also possible to identify other compounds (with a correlation greater than 0.7) such as isoamyl acetate (Figure 4c), benzaldehyde (Figure 4d), and phenylethyl acetate in the case of M4 while for M5 was possible to observe isobutyl acetate and 3-methylsulfanylprop-1-ene. These were also confirmed by an OPLS with determined compounds (phenylethanol representing M4 and methionol representing M5) (data not shown). Phenylethyl acetate is the product of the esterification of phenylethanol and acetyl-CoA enzymatic by acetyltrasferase.[33,34] Isoamyl acetate is the enzymatic esterification product of acetyl-CoA with isoamyl alcohol. Isoamyl alcohol is produced from leucine, via the Ehrlich pathway or from α-ketoisocaproic acid through the anabolic pathway.[30,31] As expected, the production of both acetate esters increased dramatically in M4, as the amino acid precursors were present in great quantities.[35] The production of benzaldehyde (Figure 4e) by *Saccharomyces cerevisiae* was previously reported by Delfini et al.[36] Thereafter, several research groups have studied the mechanisms behind that process in different microorganisms and in vitro,[37] and several metabolic pathways have been proposed. While in some species this process is enzyme catalyzed,[37] in others it is also a chemical reaction involving metal ions and O_2.[38] Based on the fermentation conditions employed in this study (relatively low temperature, acidic pH, and anaerobic conditions), some of the possibilities referred in literature may be excluded; however, the data we possess does not allow the formation of a hypothesis regarding the mechanism behind benzaldehyde formation. However, the data shows that benzaldehyde production was favored in a medium containing phenylalanine (M4), which has been suggested as a possible precursor in several studies.[36−38] Further work should be done to confirm this observation. Benzaldehyde in turn can be reduced to benzyl alcohol, explaining its presence in the same medium.[38,39]

Isobutyl acetate results from the combination of acetyl-CoA with isobutyl alcohol produced from valine, via the Ehrlich pathway or from the anabolic pathway via α-ketoisovaleric acid.[40] This compound was significantly affected by sulfur containing amino acids, an effect that was also observed, although to a lesser extent, in isoamyl acetate. In M5 neither valine nor leucine was available, thus isobutyl and isoamyl alcohol were synthesized from the corresponding α-ketoacids generated through the de novo synthesis pathway from glucose.[23] According to the literature the production of acetate esters is more closely related to acetate than to higher alcohol production.[26] Therefore, the formation of high concentrations of acetates in media supplemented with sulfur containing amino acids may be due to the high production of acetyl-CoA from cysteine. Several pathways of cysteine degradation lead to pyruvate formation[41] that is converted to acetyl-CoA via cytosolic pyruvate dehydrogenase (PDH) bypass, which is an alternative route to the PDH reaction for the conversion of pyruvate to acetyl-CoA during fermentation.[29] Further work is needed to clarify this result. 3-methylsulfanylprop-1-ene is a sulfur compound resulting from the metabolism of cysteine or methionine, the only amino acids containing sulfur atoms. The compounds observed using a targeted strategy were the same ones described in the untargeted approach, which validates this untargeted approach. The results also show that although the fermentation performance is affected by the fermentation volume, there are no scale differences concerning the quality and formation kinetics of the volatile compounds identified using the applied workflow. It would be necessary to perform further work in order to validate its applicability in complex media. Additionally, the implications of these perturbations on a sensory level would need to be validated using greater volumes.

Since the scale effect seems not to affect the quality of the volatile fraction, this workflow may lead to the discovery of new compounds or the possible modification of known pathways and showing the systemic response to perturbations. To check this hypothesis, the correlation between the coexpression of metabolites were analyzed for the 5 mL data set in the matrix generated by MarkerLynx. In this study, these correlations did not identify the presence any new compounds. The highest correlations observed were between metabolically related volatile compounds, for example, ethyl hexanoate and ethyl octanoate (0.998), isobutyl acetate and ethyl octanoate (0.985), ethyl thiolacetate and ethyl octanoate (0.984). No negative correlations were found, which might be due to the duration of the period monitored. More alterations would have occurred over the time, changing the ultimate chemical profile.

Time Profile of Volatile Synthesis. The high throughput procedure presented in this study allowed the acquisition of snapshots of the biochemical events related to the fermentation process. The linking of the kinetic profile of each volatile

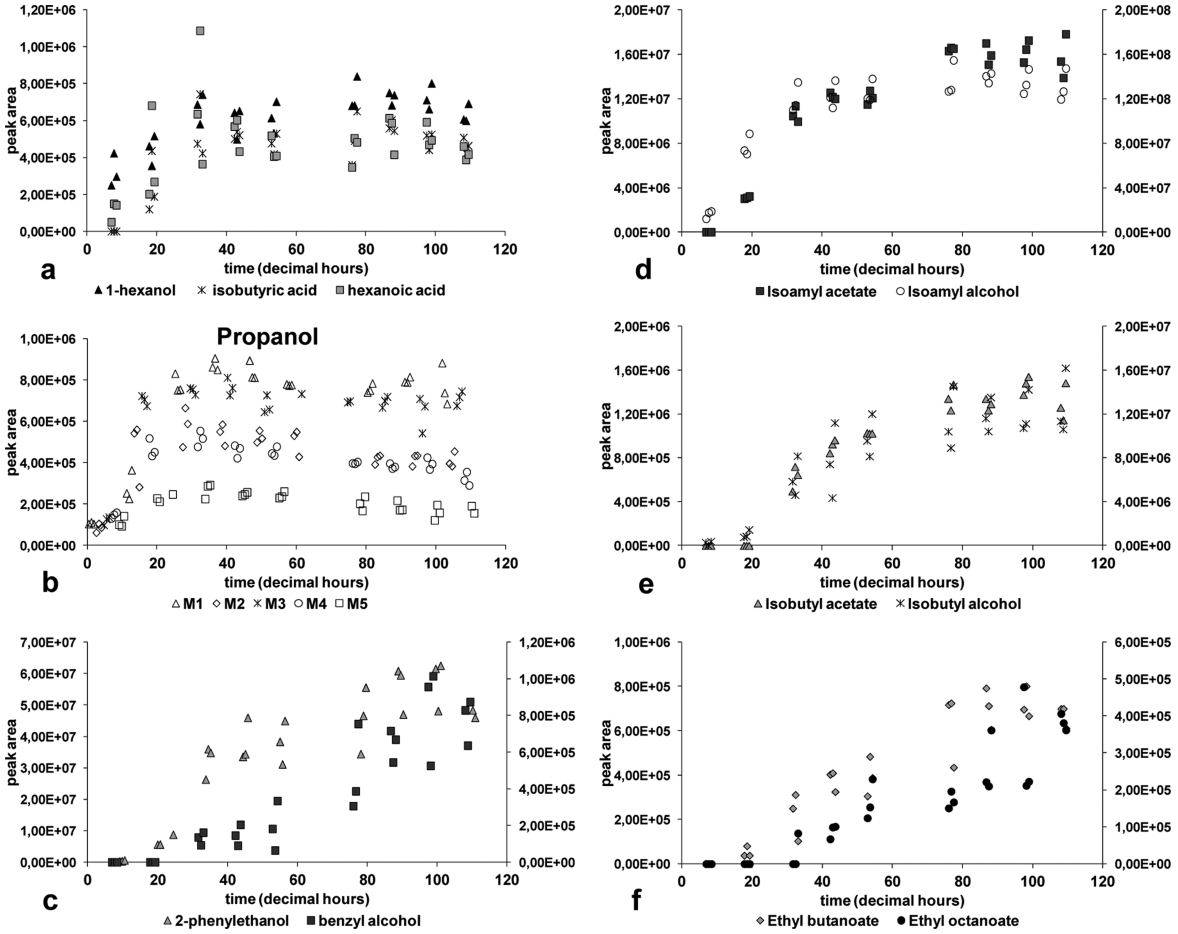

Figure 5. Kinetic profile of (a) 1-hexanol, isobutyric, and hexanoic acid in M4, (b) propanol in five media (M1−5), (c) 2-phenylethanol and benzyl alcohol in M4, (d) isoamyl acetate and isoamyl alcohol in M4, (e) isobutyl acetate and isobutyl alcohol in M4, (f) ethyl butanoate and ethyl octanoate in M4.

compound obtained in "real time" by GC-MS for 5 mL fermentations and the corresponding yeast growth curves obtained by OD measuring at 600 nm (Figure 2c) enabled the establishment of the metabolic time profile of the volatile fraction. A detailed description of the M4 data follows, but a similar approach could be done for each of the five media. During the first 20 h after the inoculation, during the lag phase, yeasts are adapting to the new media conditions, so there is no increase in the yeast population (OD remains unchanged). Although no growth occurs, yeast immediately begins to metabolize sugars and other nutrients present in media with consequent production of ethanol. There are several metabolites whose concentration start to increase during this phase namely hexanol, propanol, isobutyric, and hexanoic acids (Figure 5a). Hexanol is usually formed during the prefermentative stage of winemaking and is believed to be reductively formed by yeast from hexanal, which in turn is formed from linoleic acid.[42] Propanol reach maximum concentration few hours after inoculation and in agreement with the literature its increase was greater in the media without amino acids[43] and lower in the media containing only sulfur containing amino acids which may be related to the negative relationship between *n*-propanol production and H_2S formation from methionine and cysteine metabolism (Figure 5b).[44,45] After an adaptation period, yeast start growing. This exponential growth phase is influenced by several factors such as temperature, concentration of ammonia, amino acids, and other nutrients, and also by the oxygen level. During this phase yeasts use all the available nutrients to obtain energy and increase their population, which may reach 10^7-10^8 cells/mL. At the beginning of the exponential growth phase the concentration of higher alcohols and acids resulting either directly from sugars or from amino acid metabolism trough the Ehrlich pathway starts to increase or in some cases has already reached its maximum due to the yeast's high demand for new proteins needed for growth and reproduction. Isoamyl alcohol and isobutyric acid reached maximum concentrations about 24 h after inoculation and their concentrations remained constant (Figure 5a, d). The concentrations of isobutanol (Figure 5e), 2-phenylethanol, and benzyl alcohol (Figure 5c) started to increase at the beginning of this stage and their concentration continued increasing for the period monitored, indicating a possible continued increase in their production during the stationary phase.

During fermentation two groups of esters are synthesized: acetate esters and ethyl fatty acid esters. The synthesis of acetate esters isoamyl acetate and isobutyl acetate starts when their substrates, isoamyl alcohol, and isobutyl alcohol, are available in the media, and their time of expression is parallel to that of their precursor alcohols (Figures 5d, e).

The synthesis of ethyl fatty acid esters (ethyl hexanoate, ethyl octanoate, and ethyl decanoate) only started almost toward the end of the exponential growth phase (Figure 5f). According to literature the rate of ethyl ester formation is influenced by the

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concentrations of the two cosubstrates (acyl coenzyme A component and ethanol) and the activity of the enzymes involved in their synthesis and hydrolysis (e.g., acyl-CoA: ethanol *O*-acyltransferases, Eeb1 and Eht1). It has been suggested that the fatty acid precursor level rather than the activity of the biosynthetic enzymes is the major limiting factor for ethyl ester production.[46] Ethyl butanoate is formed from ethanol and propanoic acid derived from α-ketobutyrate,[47] an intermediate in the metabolism of several amino acids. Unlike other ethyl fatty acid esters, its concentration rises continuously from the beginning of the exponential growth period, probably due to the availability of both cosubstrates in the media since the beginning of the fermentation process (Figure 5f). After this period, yeast stops growing probably due to some nutrient deficiencies and the toxic effects of ethanol and other substances produced during alcoholic fermentation, resulting in a stationary phase. Finally yeast start dying and the population gradually decreases (decline phase).

The proposed methodology proved to be a useful tool capable of generating new insights concerning the implications of a given nutrient on the final product's volatile fraction. This knowledge can be quite useful for wine industry since it allows the possibility of modifying wine aroma by either increasing the desirable or eliminating the undesirable odors. This work culminates with the development a new metabolic profiling pipeline that relies on an unsupervised and untargeted approach applied to data acquired by a noninvasive methodology (HS-SPME/GC-MS) utilizing a small fermentation volume which allows the high throughput analyses that enables "real time" monitoring of the metabolic changes inherent to the biochemical dynamics of a perturbed complex biological system and the extraction of molecular candidates that are latter validated on its biochemical context. The pipeline proposed has several practical applications such as yeast phenotyping studies or exploring the impact of nutrient availability on yeast metabolism and subsequent volatile fraction quality. These results do require sensory validation on a larger scale.

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Notes

The authors declare no competing financial interest.

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