Determination of hydroxytyrosol produced by winemaking yeasts during alcoholic fermentation using a validated UHPLC-HRMS method

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Abstract

Hydroxytyrosol (HT) is a phenolic compound of recognized bioactivity that has been described in wines but little is known about its origin. This work demonstrates that yeast involved in wine making, i.e. *Saccharomyces cerevisiae* strains and the non-*Saccharomyces Torulaspora delbruekii*, can synthesise HT, as this compound was identified in the intracellular media of three strains by means of a developed and validated UHPLC-HRMS method with LOQ and LOD of 0.108 and 0.035 ng mL^{-1} respectively. Controlled fermentations were performed with different varieties of grapes (*Corredera*, *Moscatel*, *Chardonnay*, *Palomino fino*, *Sauvignon Blanc*, *Vijiriega*, and *Tempranillo)* and synthetic must. The *Saccharomyces cerevisiae* strain QA23 was the most efficient producer of HT from tested yeasts. On the other hand, the grape variety influences HT wine concentrations. Furthermore, the maximum concentration of HT is reached between the fourth and sixth day of fermentation. This work reveals that yeasts have a great potential for the production of HT.

Keywords: *Saccharomyces*, phenolic compound, intracellular, winemaking, Q-exactive, biomass.

1. Introduction

Hydroxytyrosol (HT) 2-(3, 4-dihydroxy-phenyl) ethanol (3, 4-DHPEA), is a higher alcohol (phenyl ethyl alcohol), found in extra-virgin olive (Fernández-Mar, Mateos, García-Parrilla, Puertas, & Cantos-Villar, 2012) as well as in in fermented beverages such as wine (Bordiga et al., 2016). The most common synthesis pathway includes the hydroxylation of its immediate precursor, tyrosol, in the Ehrlich pathway (Figure 1). This is in turn produced from tyrosine by yeasts during alcoholic fermentation (AF), as follows: (1) transamination of tyrosine; (2) decaboxylation of *p*-hydroxyphenylpyruvate by pyruvate decarboxylase; (3) reduction of *p*-hydroxyphenylaldehyde by alcohol dehydrogenase (ADH) (Pineiro, Daran, van Maris, Pronk, & Dickinson, 2008; Pineiro, Cantos-Villar, Palma, & Puertas, 2011; Satoh, Tajima, Munekata, Keasling, & Lee, 2012). Consequently, Tyrosol and HT could be considered secondary metabolites produced from tyrosine by some yeast strains by means of a transformation of amino acids (Garrido & Borges, 2013) during alcoholic fermentation (Zhu et al., 2011). On the one hand, the content of HT appears to be related mainly to the nitrogen content in musts during alcoholic fermentation while on the other, it is well known that *Saccharomyces cerevisiae* can use tyrosine and tryptophan as a source of cellular nitrogen. Fusel alcohol such as tyrosol, HT and tryptophol respectively are the main products of its catabolism (Bordiga et al., 2016). This suggests that the final contents of HT and tyrosol in wine could be influenced by microbial activity during alcoholic fermentation (Romboli, Mangani, Buscioni, Granchi, & Vincenzini, 2015).

As a bioactive compound, HT has been object of study in many research reports. Some studies have demonstrated that HT is a potent stimulator of mitochondrial biogenesis in retinal epithelial cells that contribute to eye health (Zhu et al., 2010) as well as having anticarcinogenic (Roleira et al., 2015), cardioprotective (Mnafgui et al., 2015), antidiabetic, and neuroprotective qualities (Fernández-Mar, Mateos, García-Parrilla, Puertas, & Cantos-Villar, 2012; Marhuenda et al., 2016; Rigacci & Stefani, 2016). Furthermore, the EFSA (European Food Safety Authority), has admitted a claim on these healthy effects based on the protection of LDL

particles from oxidative damage and maintenance of normal blood HDL-cholesterol concentrations, as these effects were demonstrated after the consumption of extra-virgin olive oil due to its high content in HT (European Food Safety Authority [EFSA] Panel on Dietetic Products Nutrition and Allergies [NDA], 2011).

To identity, quantify, and elucidate the occurrence of HT, different analytical methods have been used in various food matrices. Thus HT has been analysed by: gas and liquid chromatography; capillary electrophoresis in wines (Piñeiro, Cantos-Villar, Palma, & Puertas, 2011); nuclear magnetic resonance in herbal medicine products (Lemonakis, Gikas, Halabalaki, & Skaltsounis, 2013). Associated techniques have also been used, such as: HPLC with GC in olive mill wastewaters (Allouche, Damak, Ellouz, & Sayadi, 2004); HPLC with fluorescence in grape leaves (Vrhovsek et al., 2012); HPLC with DAD and with MS in wines (Boselli, Minardi, Giomo, & Frega, 2006). The use of HRMS associated with UHPLC has been used before in herbal medicinal products in the determination of HT (Lemonakis, Skaltsounis, Tsarbopoulos, & Gikas, 2016). This latter technique is a powerful tool to unequivocally identify and quantify compounds in different matrices, and therefore it may be useful in trying to elucidate the origin and evolution of HT in wines.

The aim of the present work is to develop and validate an UHPLC-HRMS method to assess HT and to investigate the origin of its occurrence in wines. For this purpose, the role of different strains of winemaking yeasts on the occurrence of HT in wines is studied during alcoholic fermentation (AF). Finally, the main objective is to examine the production of HT by yeasts through the evidence that the determination of this compound in the intracellular compartment provides when using a validated HRMS method coupled with UHPLC.

2. Materials and methods

2.1. Reagents and materials

HT standard (98%) was purchased from Chengdu Biopurify Phytochemicals Ltd. (Wenjiang Zone,Chengdu, Sichuan, China), HPLC-grade methanol was acquired from Merck (Darmstadt, Germany) and HPLC-grade formic acid from Panreac (Barcelona, Spain).

2.2. Yeast strains

The experiments on white musts were performed with the commercial wine yeast strain Enartis Ferm Aroma White (Enartis). In the fermentation of *Tempranillo* must and synthetic must, three different commercial strains: were used *S. cerevisiae* Lalvin YSEO QA23® (Lallemand), *S. cerevisiae* Red Fruit RF® (Enartis) and *T. delbrueckii* TD291 Biodiva™ (Lallemand).

2.3. Samples

2.3.1. Grapevine

Vines were grown in a typical soil-type (*albariza)* with a plant density of 3600 vines/ha. The grapes used in the fermentations belonged to an experimental cultivar located in the Rancho de la Merced (IFAPA, Jerez de la Frontera, Spain), and were from seven varieties as follows: *Corredera*, *Moscatel*, *Chardonnay*, *Palomino fino*, *Sauvignon Blanc*, *Vijiriega*, and *Tempranillo*.

The degree of ripeness was followed weekly during the maturation process (data not shown). Grapes were harvested at their stage of optimum maturity in August 2015.

2.3.2. Alcoholic fermentation procedure

2.3.2.1. Musts of white grapes

Grapes were harvested at optimum ripening conditions. Subsequently, they were destemmed, crushed, and pressed. Then pectolitic enzymes (2.5 mL hL^{-1}, Enartis ZYM, Italy) and SO_2 (Sulfosol, Sepsa-Enartis) were added into the must. After 24 h at 4 ºC, the must was dejuiced and placed in a 100-L steel vessel. Alcoholic fermentation (AF) was carried out and monitored in vessels by yeasting (Aroma White, Italy) at 18 ºC. AF was considered completed when the concentration of residual sugars was lower than 3 g L^{-1}.

2.3.2.1. Musts of *Tempranillo* grapes

Tempranillo grapes at optimum ripeness were harvested manually in 18 kg plastic boxes; they were in good sanitary conditions and were transported to the experimental winery. Musts were produced using a pneumatic press with pectolitic enzymes (3 mL hL^{-1}, Enartis ZYM, Italy) and 40 mg L^{-1} of sulphur dioxide (SO2) (Sepsa- Enartis) were added. Musts were placed in 15 stainless steel vats of 10-L capacity.

Five different methods of inoculation were used: (1) CTQA, with *Saccharomyces cerevisae* QA23 yeast strain; (2) CTRF, with *Saccharomyces cerevisae* RF yeast strain; (3) SIQA23, sequential inoculation first with commercial non-*Saccharomyces* strain *Torulaspora* delbrueckii TD291 and later when the density had decreased by 15 points just after the start of the AF with *S. cerevisiae* QA23; (4) SIRF, sequential inoculation first with commercial non-*Saccharomyces* strain *Torulaspora* delbrueckii TD291 and when density had decreased by 15 points just after the start of the AF with *S. cerevisiae* RF; and (5) SP, spontaneous fermentation without any inoculation using commercial yeasts.

2.3.3 Intracellular samples

Six alcoholic fermentations were performed in synthetic must with a sugar content of 100 g L^{-1} fructose and 100 g L^{-1} glucose and amino acids (purity ≥ 99 %) (Riou, Nicaud, Barre, & Gaillardin, 1997) with three strains of yeast (QA23, RED FRUIT and *Torulaspora delbruekii*). The must was sterilized with bottle-top vacuum filters (Nalgene PES membrane). Each Erlenmeyer flask with 750 mL of SM was inoculated with 10^6 cell mL^{-1} and capped with taps equipped with a capillary to release carbon dioxide. The fermentation was monitored by weighing the flasks daily before and after sampling.

2.3. Sampling

Samples were taken every day from inoculation until the end of AF. Samples were collected and stored at -80 ºC until the analysis. The end of AF for each grape variety was different and, consequently, each fermentation lasted a different number of days. The end of AF was reached

148 when the sugars were almost all consumed (lower 3 g L^{-1}). Table 1 shows the time of the
149 fermentation process and the concentrations of sugars at the end of alcoholic fermentation in
150 white grapes (Table 1A). Table 1B shows the time of fermentation and the concentration of
151 sugars (approximately 10.9 ºBe) at the end of fermentation of the 5 fermentation methods for
152 *Tempranillo* grapes.

153 **2.3.1. Intracellular metabolite extraction**

154 Samples of the intracellular compartment were collected at the second day of fermentation in a
155 volume corresponding to 10^9 cells mL^{-1}. Immediately, they were subjected to a cold glycerol-
156 saline quenching (Villas-Bôas & Bruheim, 2007), were stored at -80 ºC until the extraction
157 process was conducted. The intracellular extraction was performed following the method
158 reported by Smart et al. with minor modifications (Smart, Aggio, Van Houtte, & Villas-Bôas,
159 2010). To the cell pellets, 2.5 mL of cold methanol-water solution (50% [v/v], -30 ºC) were
160 added, mixed for 1 min and then frozen at -80 ºC. The samples were subjected to two cycles of
161 freeze-thaw (thaw in an ice bath for 4 min; then were frozen at -80 ºC for 30 min). After the last
162 cycle, they were subjected to sonication for 1 min in an ice bath using an ultrasonic liquid
163 processor (Sonicator Sonoplus HD 2070, Bandelin electronic GmbH & Co. KG, Berlin,
164 Germany). Afterwards, the samples were centrifuged at 36086 *g* for 20 min at -20 ºC using a
165 refrigerated centrifuge (Sorvall LYNK 6000, ThermoFisher scientific, Waltham, MA USA).
166 Another 2.5 mL of cold methanol-water was added to the pellet and then centrifuged; the
167 supernatants were collected, pooled, and stored at -80 ºC until analysed.

168 **2.4. Sample clean up**

169 Samples were cleaned up as previously reported by Rodriguez-Naranjo et al. (Rodriguez-
170 Naranjo, Gil-Izquierdo, Troncoso, Cantos, & Garcia-Parrilla, 2011) with the following
171 modifications: C18 SPE cartridges (Variant, Agilent) were conditioned with 2 mL of methanol
172 and 2 mL of milliQ water. An aliquot of 500 µL of sample was loaded followed by a washing
173 step with 2 mL of a 10% v/v methanol solution. The analytes were eluted with 1 mL of

methanol; afterwards solvents were evaporated until dryness at 34ºC, 2000 rpm during 6 h with a vacuum concentrator (HyperVAC-LITE, GYOZEN, Korea). Then samples were reconstituted with 167 µL of methanol/water 10% v/v and stored at -20 ºC until analysis.

2.3. UHPLC/HRMS parameters

The analysis was carried out in a UHPLC Dionex Ultimate 3000 system (Thermo Fisher Scientific (Bremen, Germany) all devices were controlled by Chromeleon Xpress Software. The column used was a ZORBAX RRHDSB-C18 (2.1×100 mm, 1.8-µm particle size) with a guard column (2.1×5 mm, 1.8-µm particle size). Column and guard column were purchased from Agilent Technologies (Waldbronn, Germany). The separation was performed using column temperature of 40 ºC, a flow of 0.5 mL min $^{-1}$, and injection volume of 5 µL. The chromatographic conditions consisted of two phases (A) aqueous formic solution 0.1%, and (B) solution 0.1% of formic acid in methanol and the gradient was programmed as follows: 95% A, 5% B (0 – 1 min); 0% A, 100% B (1 – 8.5 min); 95% A, 5% B (8.6 – 10 min).

A target MS^2 in negative mode with a heated ionization source HESI was selected using the transition 153 →123 HCD 100.00 in order to both identify and quantify. The main HRMS parameters were heater and capillarity temperature (400 –275 ºC respectively), spray voltage 3.0 KV; flow rates of sheath gas and auxiliary gas (65, 25 arbitrary units, respectively). Other parameters of HRMS methods were normalized collision energy (NCE) 40; S-lens RF 50% and mass resolving power (RP) 70,000 FWHM.

2.4. Statistical Analysis

Statistical analyses were performed by means of Statistica software (StatSoft, 2014). One-way analysis of variance (ANOVA) and Tukey's HSD (honest significant difference) test were assessed to test significant differences at $p < 0.05$. Additionally, principal component analysis (PCA) was used for data analysis

3. Results and discussion

3.1. Method validation

The validation procedure was carried out following different international guides (AOAC, 1998; FDA, 2012) to establish parameters, such as: detection (LOD) and quantification (LOQ) limits, precision, linearity, recovery, matrix effects, and effects of solid-phase extraction. As a blank, a fermentation sample was used after cleaned using SPE, because it bears a greater similarity to the matrix than to the must without fermentation.

The linearity, LOD, and LOQ were experimentally determined by the injection of 11 solutions in the fermentation medium cleaned using SPE, as explained in Section 2.4, because it reproduces matrix characteristics better than does the solvent (methanol/water 10% v/v). Calibration standards of HT were prepared for each analytical batch and three replicates were determined at 11 concentrations (1000, 500, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, and 0.39 ng mL^{-1}) with 4 degrees of linearity. The detection limits were calculated based on the standard deviation of the response (σ) and the slope (Ich, 2005). Calibrations curves with their slope (*S*), intercept and correlation coefficient (*r*) were calculated by plotting the peak area vs. the concentration of the standards using Statististica software version 12 (StatSoft, 2014).

$LOD = 3.3\ \sigma\ S^{-1} LOQ = 10\ \sigma\ S^{-1}$

The results indicated linearity by a curve with $r^2 = 0.9995$ with LOD 0.035 ng mL^{-1} and LOQ 0.108 ng mL^{-1}. These results improved the limits reached before by Bordiga et al. in wines using HPLC-PDA-MS/MS (LOD 4 ng mL^{-1} – LOQ 11 ng mL^{-1}) (Bordiga et al., 2016) probably because they determined several compounds simultaneously and our method focused on HT.

The intermediate precision was calculated measuring standard deviation (RSD) in a set of two concentrations (0.1 - 1 ng mL^{-1}) for 5 days with 5 replicates per concentration. Repeatability was assessed during a working session with 5 replicates per concentration. The data for intermediate precision and repeatability are shown in Table 2.

The matrix effect was tested in a clean-up matrix by spiking with standard solution in 10 concentrations: 0.079, 0.158, 0.316, 0.632; 1.264, 5.056, 20.224, 80.896, 404.48, and 1011 ng mL^{-1}.The slopes resulting from the spiked matrix and calibration solutions (methanol 10% v/v) in the linear range were used to evaluate the matrix effect. The relation between the slopes was defined as (slope in solvent/slope in the spiked matrix)*100 and expressed as %ME (Trufelli, Palma, Famiglini, & Cappiello, 2011). The resulting value was 1.05%, which is considered an irrelevant value, as the calibration curves in the solvent and matrix spiked are very similar. Figure 1 of supplementary material shows the results.

Recovery was calculated from the spiked matrix at five different concentrations ranging from 1.2 to 1011.2 ng mL^{-1}. The results were from 116 – 58% within the recommended values (40-120%) for concentrations ranging from 1 to 1000 ng mL^{-1} (Gustavo González & Ángeles Herrador, 2007). Figure 2 of supplementary material shows the results.

SPE was used as a cleaning technique because it allowed the removal of different components that could be interfering with mass analysis. A solid-phase extraction (SPE) approach has been tested in order to avoid overestimation and sub-estimation of the quantity. Three different solutions were prepared with a concentration LOQ, LOQ + 50% and 3LOQ in methanol /water 10% v/v, which were analysed after the SPE clean-up procedure (Gasperotti, Masuero, Guella, Mattivi, & Vrhovsek, 2014). The results showed an extraction efficacy of 96.7%, 86.3%, and 143.2%, respectively. On the other hand, to evaluate the amount of analyte that is dragged in the washing step, the solutions were analysed and the HT contents were under LOQ limits, indicating the clean-up procedure was efficient.

3.2. Intracellular HT

Hydroxytyrosol is a phenolic compound that could be formed from a degradation or transformation from other polyphenolic structures present in wines (i.e. anthocyanins, (Motilva et al., 2016). Likewise it is formed from oleopurein degradation in olive oil (Charoenprasert & Mitchell, 2012). Nonetheless, different synthesis of HT has been proposed. For instance, the

metabolism in humans involves a pathway starting from dopamine, which is transformed by the monoaminoxidase to give 3,4-dihydroxyphenylacetaldehyde that can be reduced by the aldehyde reductase to HT (Pérez-Mañá et al., 2015). Additionally, the Ehrlich pathway shown in Figure 1 relates the amino acid metabolism with HT synthesis. However, up to now, no direct evidence of this synthesis by yeast could be determined. To demonstrate that it is a metabolite formed by yeast, we analysed the intracellular media of the yeast, as this could unequivocally demonstrate its origin apart from others that might happen. Figure 2 shows three mass chromatograms of the biomass of the strains QA, RF and *T. delbruekii* taken at day 2 of the alcoholic fermentations of synthetic must; each of these strains were analysed in duplicate. A total of six samples were analysed and HT quantified as follows: 8.6 ± 2.7 ng mL^{-1} in the intracellular media of QA at day 2 of fermentation; 106.2 ± 35.1 ng mL^{-1} in the intracellular media of RF at day 2; and 16.1 ± 2.3 ng mL^{-1} in the intracellular media of *T. delbruekii* at day 2 of fermentation. These results demonstrate the production of HT by the strains studied, conferring yeast with a high potential as a producer of this bioactive compound.

3.3. Fermentations in *Tempranillo* must

Three fermentations with different inoculation processes were performed with the *Tempranillo* variety. As shown in Figure 3A, the production of HT was influenced by the yeast strain involved in the fermentation process. The higher concentrations of HT were observed at day 5 in CTQA and at day 3 in CTRF, ranging between 400 and 235 ng mL^{-1}, respectively. Only in the case of CTQA, was the maximum HT achieved at the moment when the reducing sugars were totally consumed. When sequential fermentation was performed, lower concentrations of HT were found, as can be seen when comparing CTQA, QA and *T delbruekii,* (41.3%; Figure 3A left). Moreover, when SP and SIQA were compared, SP fermentation was found to show higher concentrations (24.3%) over the other fermentations. On the other hand, Figure 3A (right) shows RF fermentations and it can be observed that concentration values in SIRF (8.51%) were lower than in CTRF. In addition, the content of HT in SP fermentation reached

values 23.8% higher than the concentration in SIRF and CTRF (16.7%). Therefore, our results imply that when *T. delbruekii* was used in the fermentations, the HT was in a lower concentration. Romboli et al. made a similar observation in sequential fermentation with another non-*Saccharomyces* strain, *C zemplinina* (Romboli et al., 2015). These researchers reported concentrations of 18.4 mg L^{-1} for HT+ tyrosol in wines produced by *S. cerevisiae* alone, and concentrations of 5.8 mg L^{-1} of HT+tyrosol in wines produced by sequential inoculation with *C. zemplinina* and *S. cerevisiae* Sc1.

Figure 3B shows the score plots of PCA analysis displaying on the right side the samples that were separated considering the methods of inoculation. The samples CTQA and CTRF presented the same location while the samples of sequential inoculation were separated from each other. Spontaneous fermentation was located on the bottom-left quadrant, possibly related to the influence of concentrations of days 7 and 8, which appear in the same place in the projection of cases due to their high concentrations of SP samples.

3.4. Fermentations in must of six white varieties of grapes

To study the effect of different white grapes on HT production during alcoholic fermentation, musts made from six white grape varieties were analysed after alcoholic fermentation by *S. cerevisiae* strain Aroma White. The grapes varieties were *Corredera*, *Moscatel*, *Chardonnay*, *Sauvignon Blanc*, *Palomino Fino* and *Vijiriega*, and the results are represented in a bar graph together with reducing sugars (Figure 4A). All varieties followed a similar trend, a progressive increase until the highest concentration was reached at the fifth day (173, 159, 167, 288, 89 and 238 ng mL^{-1}, respectively) except for the variety *Chardonnay*, which presented a slight delay, and reached the highest concentration one day later (185 ng mL^{-1}). The maximum contents of HT were determined when the value of reducing sugars ranged from 53% to 35% of the initial concentration. After this point, the concentrations decreased, falling to the previous values achieved on the 3rd and 4th days (no significant differences $p < 0.05$). In fact, all these results show that the time course of hydroxytyrosol production by the AROMA WHITE strain was

affected by the grape-must composition and, consequently, by the duration of the fermentation. These results agree well with those reported before by Romboli et al. (2015), who correlated high amounts of HT with the slowness in the fermentation process. In fact, the longer the fermentation lasted, the higher the HT concentration, as with the fermentations of *Sauvignon Blanc*.

Figure 4B plots the PCA analysis, representing on the left the days of the fermentation process. The data are grouped into four clusters, two smaller for the first two days (day 1and 2) and other of last days (12, 13, 14, and 15), located on the right side of the plot. The biggest cluster is in the middle of the plot, corresponding to the interval from the 8^{th} to the 11^{th} days and day 3. On the left, the cluster corresponds to the interval from days 4 to 7, which are those with the highest concentrations. Figure 4B displays the projection of the varieties of grapes treated as variables (Figure 4B right). However, the Chardonnay variety is located at the bottom of the projection, probably being influenced by the location of the intermediate cluster (3, 8, 9, 10, and 11) days in which the HT content remained practically unchanged (159 to 134 ng mL^{-1}). Although further studies are required to understand how the chemical composition of the must influences the HT concentration as well as the role that this compound exerts on the yeast, it is clear from our results that the strains studied synthesise HT and therefore this compound is found in wines.

4. Conclusions

As we know, this is the first study available in which HT has been identified and quantified in an intracellular compartment of *Saccharomyces* (QA23, RF) and Non-*Saccharomyces* (*Torulaspora delbruekii*). This could be accomplished thanks to a validated HRMS method developed specifically to diminish LOD and LOQ.

Furthermore, we ascertained that the strain is a crucial factor that influences the production of HT in wines. *Saccharomyces cerevisiae* (QA23) was a more efficient producer of HT than RF in grape musts. Sequential fermentations involving the non-*Saccharomyces* yeast, *T. delbruekii*, adversely affect the content of HT.

Additionally, the composition of grape must affect the HT concentration. *Sauvignon Blanc* and *Vijiriega* were grape varieties from which AROMA WHITE produced higher concentrations at 5th day of alcoholic fermentation. In summary, HT was a bioactive compound produced by yeast strain that can be modulated both by the involved strain and by the composition of the must.

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References

Allouche, N., Damak, M., Ellouz, R., & Sayadi, S. (2004). Use of Whole Cells of Pseudomonas aeruginosa for Synthesis of the Antioxidant Hydroxytyrosol via Conversion of Tyrosol. *Applied and Environmental Microbiology*, 70(4), 2105–2109.

AOAC, A. (1998). Peer-Verified Methods Program, Manual on Policies and Procedures. AOAC, Arlington, VA, 1–38.

Bordiga, M., Lorenzo, C., Pardo, F., Salinas, M. R., Travaglia, F., Arlorio, M., Coïsson, J.D, Garde-Cerdán, T. (2016). Factors influencing the formation of histaminol, hydroxytyrosol, tyrosol, and tryptophol in wine: Temperature, alcoholic degree, and amino acids concentration. *Food Chemistry*, 197 Pt B, 1038–45.

Boselli, E., Minardi, M., Giomo, A., & Frega, N. G. (2006). Phenolic composition and quality of white d.o.c. wines from Marche (Italy). *Analytica Chimica Acta*, 563(1–2 SPEC. ISS.), 93–100.

Charoenprasert, S., & Mitchell, A. (2012). Factors influencing phenolic compounds in table olives (Olea europaea). Journal of Agricultural and Food Chemistry, 60(29), 7081–7095.

European Food Safety Authority (EFSA) Panel on Dietetic Products Nutrition and Allergies (NDA). (2011). Scientific Opinion on the substantiation of health claims related to polyphenols in olive and protection of LDL particles from oxidative damage. EFSA Journal, 9(1924), 1–25.

FDA. (2012). Guidelines for the Validation of Chemical Methods for the FDA Foods Program. Foods Program Science and Research Steering Committee, 37.

Fernández-Mar, M. I., Mateos, R., García-Parrilla, M. C., Puertas, B., & Cantos-Villar, E. (2012). Bioactive compounds in wine: Resveratrol, hydroxytyrosol and melatonin: A review. *Food Chemistry*, *130*(4), 797–813.

375 Garrido, J., & Borges, F. (2013). Wine and grape polyphenols — A chemical perspective. *Food*
376 *Research International*, 54(2), 1844–1858.

377 Gasperotti, M., Masuero, D., Guella, G., Mattivi, F., & Vrhovsek, U. (2014). Development of a
378 targeted method for twenty-three metabolites related to polyphenol gut microbial
379 metabolism in biological samples, using SPE and UHPLC-ESI-MS/MS. *Talanta*, 128,
380 221–30.

381 Gustavo González, A., & Ángeles Herrador, M. (2007). A practical guide to analytical method
382 validation, including measurement uncertainty and accuracy profiles. *TrAC Trends in*
383 *Analytical Chemistry*, 26(3), 227–238.

384 Hazelwood, L. A., Daran, J.-M., van Maris, A. J. A., Pronk, J. T., & Dickinson, J. R. (2008).
385 The Ehrlich pathway for fusel alcohol production: a century of research on Saccharomyces
386 cerevisiae metabolism. *Applied and Environmental Microbiology*, 74(8), 2259–66.

387 Ich. (2005). ICH Topic Q2 (R1) Validation of Analytical Procedures : Text and Methodology.
388 *International Conference on Harmonization*, 1994(November 1996), 17.

389 Lemonakis, N., Gikas, E., Halabalaki, M., & Skaltsounis, A. L. (2013). Development and
390 Validation of a Combined Methodology for Assessing the Total Quality Control of Herbal
391 Medicinal Products - Application to Oleuropein Preparations. *PLoS ONE*, 8(10), 1–12.

392 Lemonakis, N., Skaltsounis, A.-L., Tsarbopoulos, A., & Gikas, E. (2016). Optimization of
393 parameters affecting signal intensity in an LTQ-orbitrap in negative ion mode: A design of
394 experiments approach. *Talanta*, 147, 402–9.

395 Mnafgui, K., Hajji, R., Derbali, F., Khlif, I., Kraiem, F., Ellefi, H., Elfeki, A., Allouche, N.,
396 Gharsallah, N. (2015). Protective Effect of Hydroxytyrosol Against Cardiac Remodeling
397 After Isoproterenol-Induced Myocardial Infarction in Rat. *Cardiovascular Toxicology*,
398 147–155.

399 Marhuenda, J., Medina, S., Martinez, P., Arina, S., Zafrilla, P., Mulero, J., Oger, C., Galano, J.

M., Durand, T., Ferreres, F., and Gil-Izquierdo, A. (2016). Melatonin and hydroxytyrosol-rich wines influence the generation of DNA oxidation catabolites linked to mutagenesis after the ingestion of three types of wine by healthy volunteers. *Food Funct.*

Motilva, M. J., Macià, A., Romero, M. P., Rubió, L., Mercader, M., & González-Ferrero, C. (2016). Human bioavailability and metabolism of phenolic compounds from red wine enriched with free or nano-encapsulated phenolic extract. *Journal of Functional Foods*, *25*, 80–93.

Pérez-Mañá, C., Farré, M., Rodríguez-Morató, J., Papaseit, E., Pujadas, M., Fitó, M., Robledo, P.,Covas, M. I., Cheynier, V., Meudec, E., Escudier, J. L., de la Torre, R. (2015). Moderate consumption of wine, through both its phenolic compounds and alcohol content, promotes hydroxytyrosol endogenous generation in humans. A randomized controlled trial. *Molecular Nutrition and Food Research*, 59, 1213-1216

Pineiro, Z., Cantos-Villar, E., Palma, M., & Puertas, B. (2011). Direct Liquid Chromatography Method for the Simultaneous Quantification of Hydroxytyrosol and Tyrosol in Red Wines. *Journal of Agricultural and Food Chemistry*, 59(21), 11683–11689.

Riou, C., Nicaud, J. M., Barre, P., & Gaillardin, C. (1997). Stationary-phase gene expression in Saccharomyces cerevisiae during wine fermentation. *Yeast*, 13, 903–915.

Rodriguez-Naranjo, M. I., Gil-Izquierdo, A., Troncoso, A. M., Cantos, E., & Garcia-Parrilla, M. C. (2011). Melatonin: A new bioactive compound in wine. *Journal of Food Composition and Analysis*, 24(4–5), 603–608.

Roleira, F. M. F., Tavares-Da-Silva, E. J., Varela, C. L., Costa, S. C., Silva, T., Garrido, J., & Borges, F. (2015). Plant derived and dietary phenolic antioxidants: Anticancer properties. *Food Chemistry*, 183, 235–258.

Romboli, Y., Mangani, S., Buscioni, G., Granchi, L., & Vincenzini, M. (2015). Effect of Saccharomyces cerevisiae and Candida zemplinina on quercetin, vitisin A and

425 hydroxytyrosol contents in Sangiovese wines. *World Journal of Microbiology &*
426 *Biotechnology*, 31(7), 1137–45.

427 Satoh, Y., Tajima, K., Munekata, M., Keasling, J. D., & Lee, T. S. (2012). Engineering of a
428 tyrosol-producing pathway, utilizing simple sugar and the central metabolic tyrosine, in
429 Escherichia coli. *Journal of Agricultural and Food Chemistry*, 60(4), 979–84.

430 Smart, K. F., Aggio, R. B. M., Van Houtte, J. R., & Villas-Bôas, S. G. (2010). Analytical
431 platform for metabolome analysis of microbial cells using methyl chloroformate
432 derivatization followed by gas chromatography-mass spectrometry. *Nature Protocols*,
433 5(10), 1709–1729.

434 StatSoft, Inc. (2013). Electronic Statistics Textbook. Tulsa, OK: StatSoft. WEB:
435 http://www.statsoft.com/textbook/.

436 Trufelli, H., Palma, P., Famiglini, G., & Cappiello, A. (2011). An Overview of matrix effects in
437 liquid chromatography-mass spectrometry. *Mass Spectrometry Reviews*, (30), 491–509.

438 Villas-Bôas, S. G., & Bruheim, P. (2007). Cold glycerol–saline: The promising quenching
439 solution for accurate intracellular metabolite analysis of microbial cells. *Analytical*
440 *Biochemistry*, 370(1), 87–97.

441 Vrhovsek, U., Malacarne, G., Masuero, D., Zulini, L., Guella, G., Stefanini, M., Velasco, R.
442 Mattivi, F. (2012). Profiling and accurate quantification of trans-resveratrol, trans-piceid,
443 trans-pterostilbene and 11 viniferins induced by Plasmopara viticola in partially resistant
444 grapevine leaves. *Australian Journal of Grape and Wine Research*, 18(1), 11–19.

445 Zhu, L., Liu, Z., Feng, Z., Hao, J., Shen, W., Li, X., Sunc, L, Sharmand, E., Wange, Y., Wetze,
446 K., Webere, P., Xianglin, S., Liu, J. (2010). Hydroxytyrosol protects against oxidative
447 damage by simultaneous activation of mitochondrial biogenesis and phase II detoxifying
448 enzyme systems in retinal pigment epithelial cells. *Journal of Nutritional Biochemistry*,
449 21(11), 1089–1098.

Table 1A. Sampling and mean of reducing sugars content at the end of fermentation in different variety of grapes

Grape variety	*Palomino fino*	*Vijiriega*	*Corredera*	*Moscatel*	*Sauvignon Blanc*	*Chardonay*
Days of fermentation	7	10	11	11	13	15
Reducing Sugars (g L^{-1})	2.00	1.30	2.33	1.47	1.55	1.95

Table 1B. Sampling days and mean of the content of reducing sugars at the end of fermentation in different inoculations ways.

Fermentation	*CTQA23*	*CTRF*	*ISQA23*	*ISRF*	*Spontaneous*
Days of fermentation	13	13	14	15	13
Reducing Sugars (g L^{-1})	0.64	1.02	0.56	0.84	1.2

Table 2A. Parameters of calibration curves. LDR (linear dynamic range), LOD (limit of detection), LOQ (limit of quantitate).

				Curve		
	LDR	LOD	LOQ	(slope)	(offset)	R^2
Matrix-spiked calibration	0.079-1011.00	0.03	0.01	950135	-5070483	0.9991
Solvent calibration (methanol 10%)	0.079-1011.00	0.03	0.08	72224	-355656	0.9991

Table 2B. Accuracy and repetitivity.

	Intra-day (%RSD)	Inter-day (%RSD)	Accuracy (%RE)
Low concentration	1.6	0.5	-1
High concentration	1.4	0.5	-1

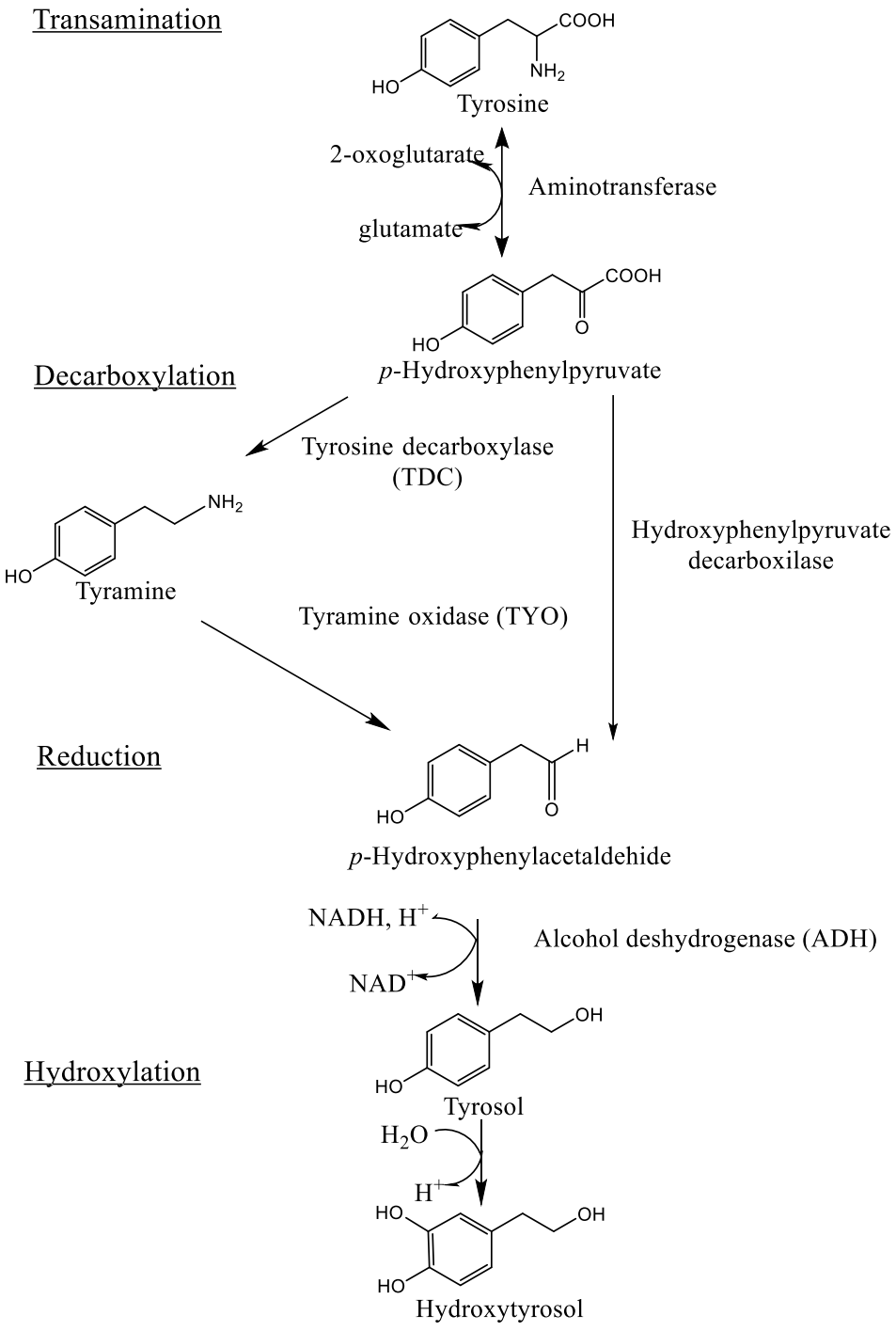

Figure 1. Ehrlich pathway for the production of hydroxytyrosol

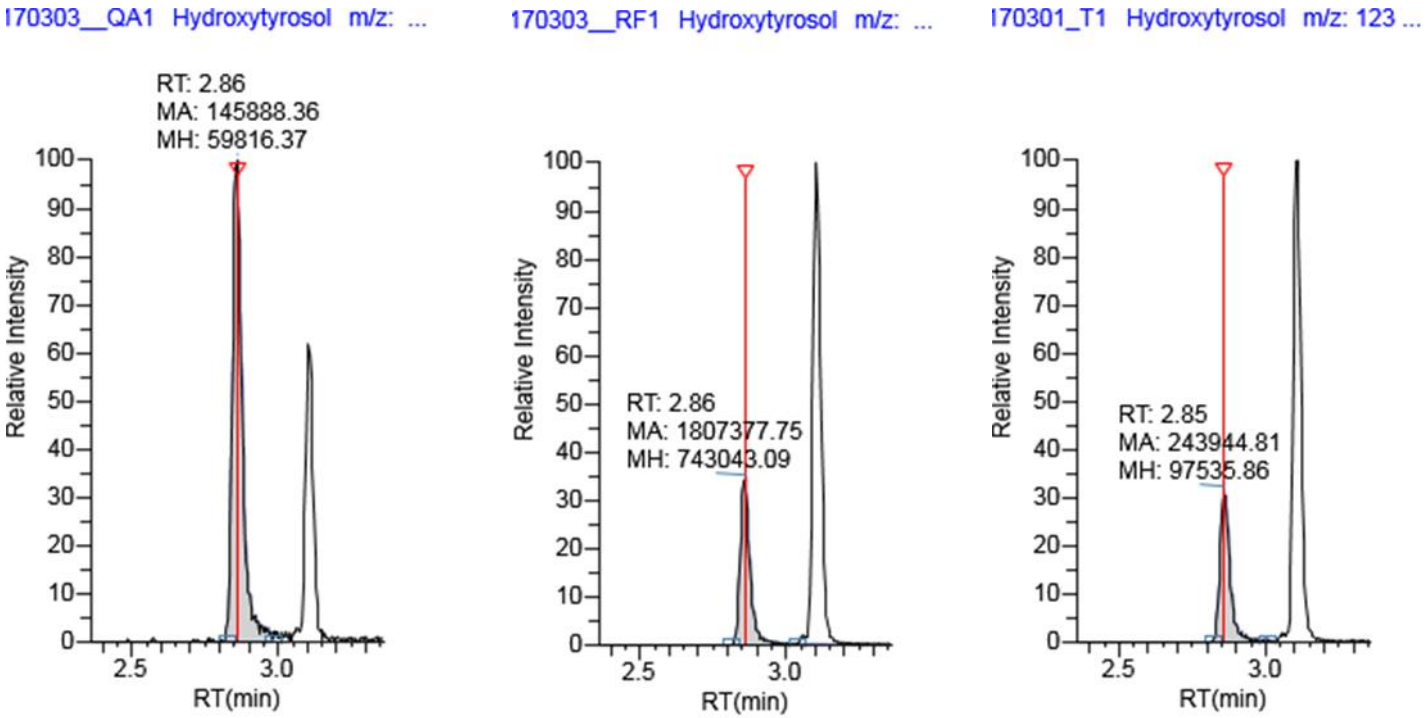

Figure 2. TIC chromatogram of HT of intracellular samples of second day of fermentation. On the left the QA sample, in the middle RF and on the right *T. delbruekii*.

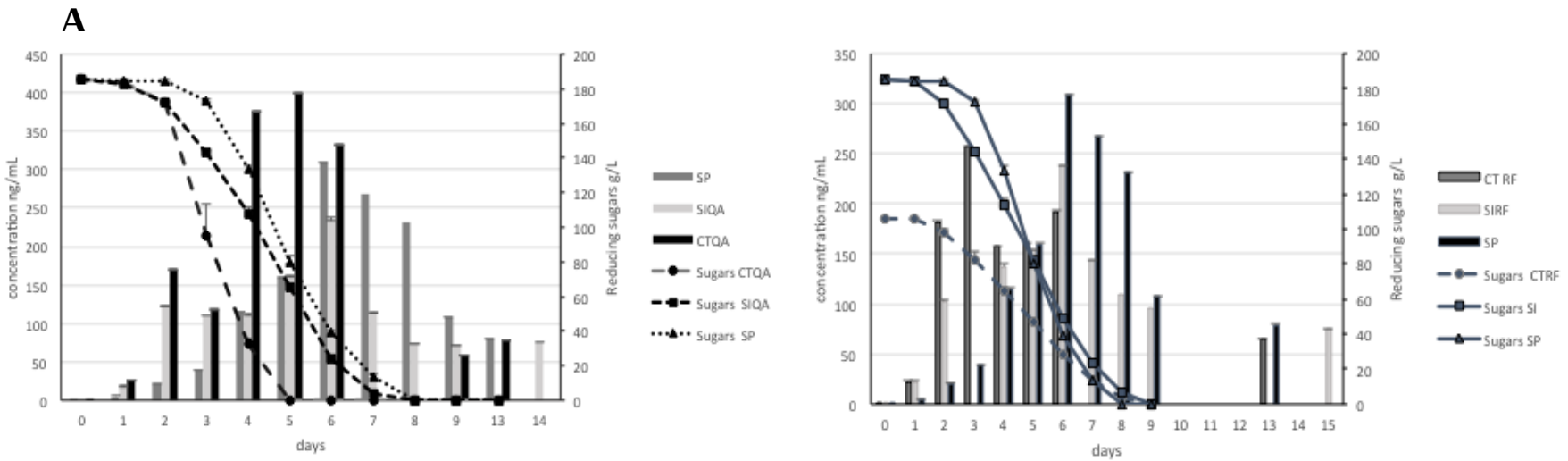

Figure 3A. Effects of two yeast strains (QA23 on the left and RF on the right) on the production of hydroxytyrosol, and time course reducing sugar in the fermentation of natural must of Tempranillo grapes. Different forms of inoculation were used: SI (sequential inoculation). SP (spontaneous fermentation). CTQA (control fermentation with *Saccharomyces cerevisiae* QA23); CTRF (control fermentation with RED FRUIT).

B

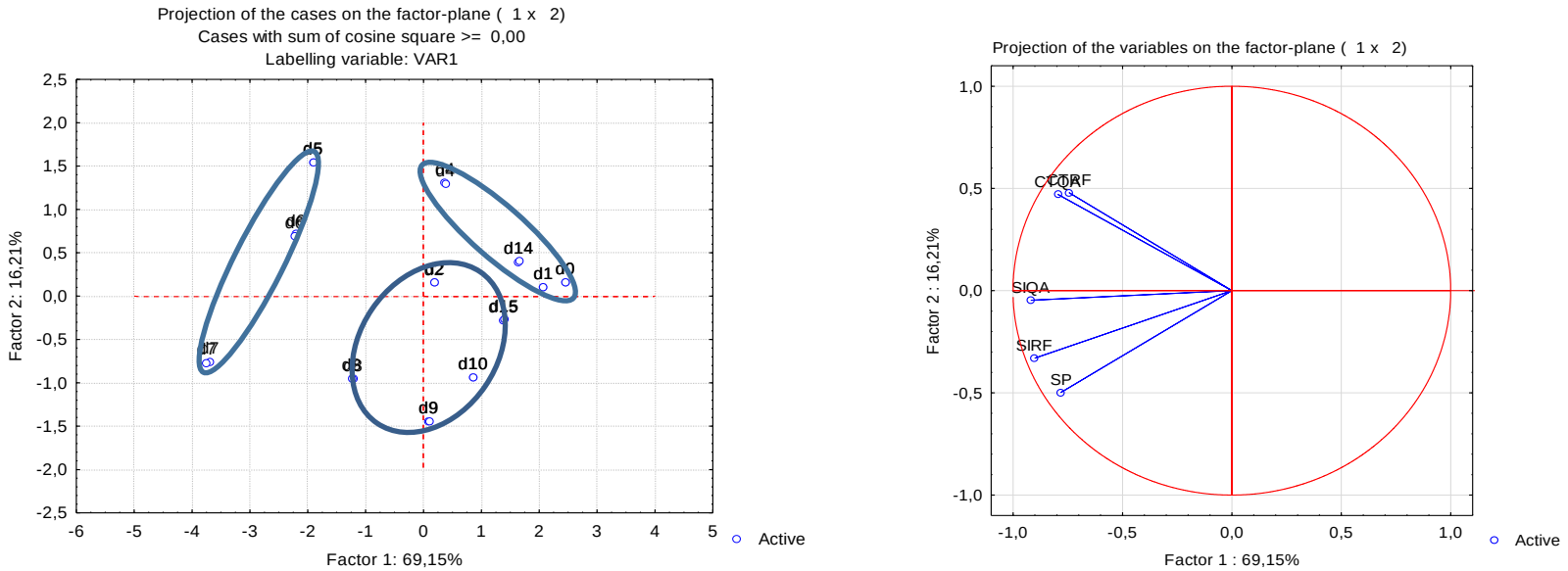

Figure 3B. Score plot of PCA analysis, on the left side, considering days of fermentation and on the right side considering different methods of inoculation

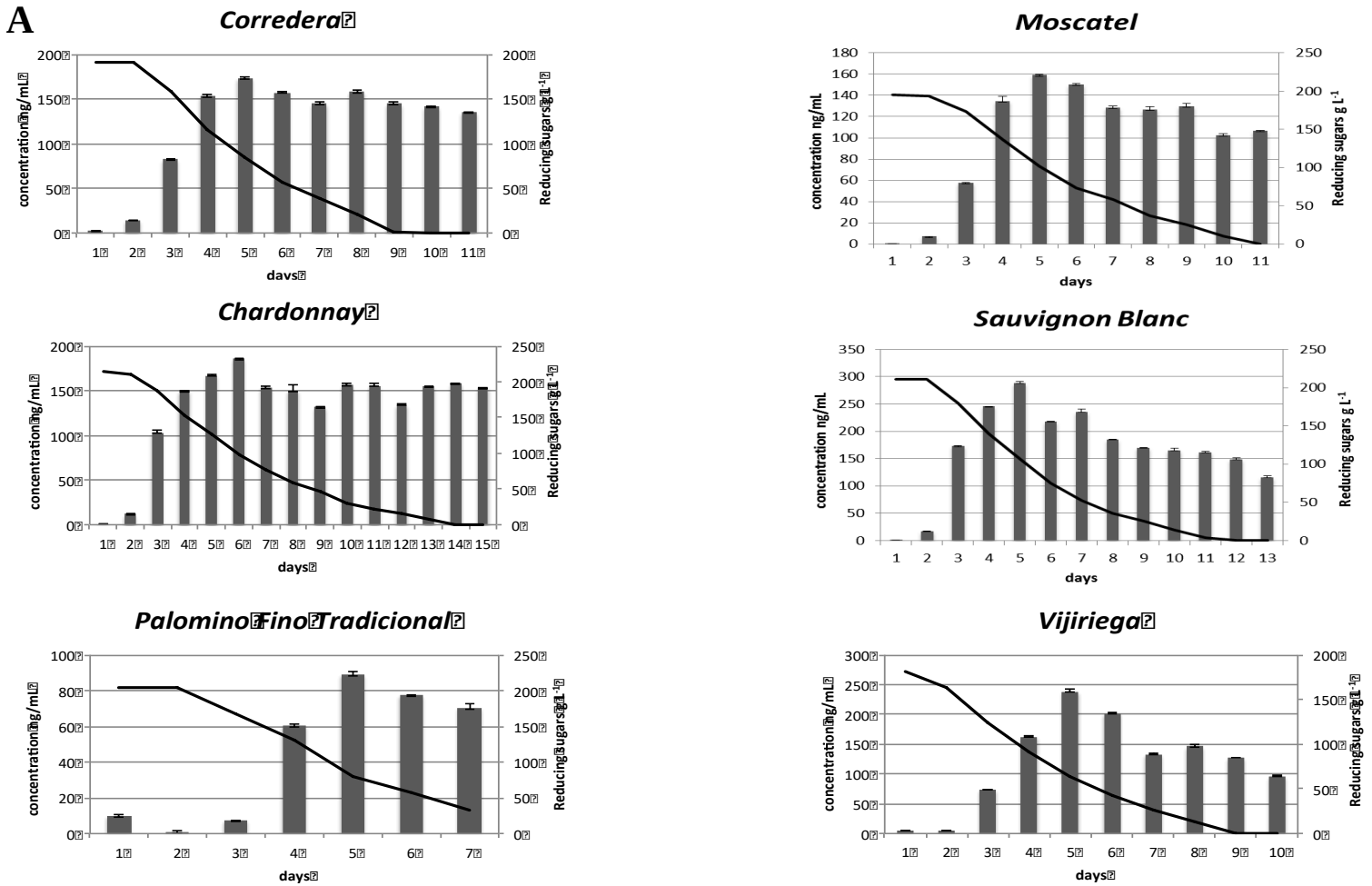

Figure 4A. Time course of HT and consumption of reducing sugars in alcoholic fermentation in natural must of six different grape varieties.

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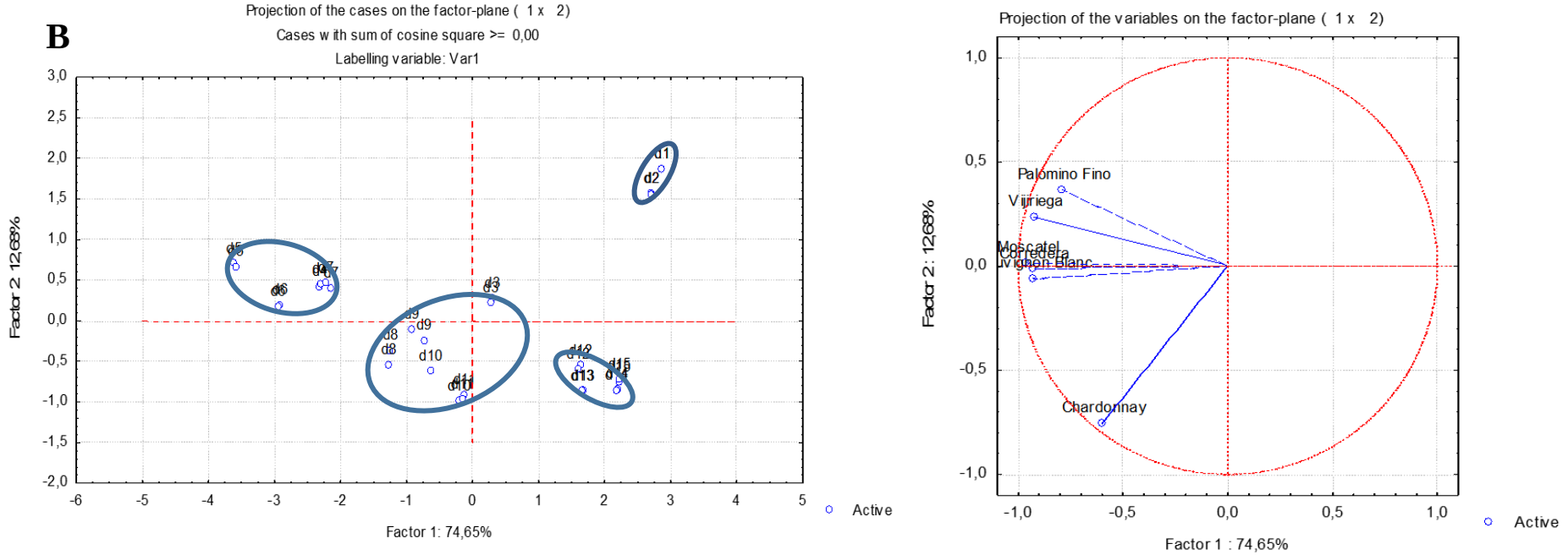

Figure 4B. Score plot of PCA analysis, on the left side considering days of fermentation and on the right considering the varieties of grapes using in the elaboration of musts.