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Chemical and Biochemical Change of Healthy Phenolic Fractions in Winegrape by Means of Postharvest Dehydration

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Clusters of Aleatico winegrape were picked at 18 °Brix and placed at 10, 20, or 30 °C, 45% relative humidity (RH) and 1.5 m/s of air flow to dehydrate the berries up to 40% of loss of initial fresh weight. Sampling was done at 0, 10, 20, 30, and 40% weight loss (wl). Selected polyphenols and sugar content (expressed as SSC = soluble solids content) both measured on dry weight basis, polyphenol oxidase (PPO), and phenylpropanoid pathway gene expression were analyzed. Phenolic acids increased significantly at 20% wl at 20 °C, while at 10 °C the increase was lower. Stilbenes (*trans*-resveratrol and *trans*-piceid) and catechins rose more than double to 100 mg/kg and more than 3-fold to 135 mg/kg at 20 °C and 10% wl. At 10 °C the increase of these compounds was less, but higher than initial values. At 30 °C, except for a significant rise at 10% wl for catechins and stilbenes, all the rest of the compounds diminished. Anthocyanins increased at 10 and 20 °C, but decreased at 30 °C. PPO rapidly increased at 20 and 30 °C at 10% wl and then declined, while at 10 °C the activity lasted longer. Relative gene expression of phenylalanine ammonia lyase (PAL), stilbene synthase (STS), chalcone isomerase (CHI), dihydroflavonol reductase (DFR) were upregulated at 10 °C more than at 20 °C, at 20% wl, while at 30 °C the gene expression was downregulated.

KEYWORDS: Grape; water loss; temperature; polyphenols; gene expression

INTRODUCTION

Increasing polyphenol content in wine is a common objective of wine producers for quality purposes. On the other hand, recently the interest for these compounds has become even greater because of their nutraceutical effect. Regular, moderate consumption of red wine is linked to a reduced risk of coronary heart disease and to lower overall mortality (*1*). High consumption of wine increases high density lipoprotein cholesterol, but, in contrast, coronary artery disease (CAD) is increased by high alcohol consumption (*2*). The positive effect of grape derived polyphenols as stimulators of the endothelial formation of vasoprotective factors is well-known (*3*), and, more specifically, grape compounds such as oligomeric procyanidin (*4*) have been shown to suppress the synthesis of endothelin-1 (ET-1), a peptide with a vasoconstricting effect. Moreover, we know that *trans*-resveratrol has positive control on intestinal cancer development (*5*, *6*); it also inhibits the production of reactive oxygen species (ROS) and, ultimately, platelet function (*7*). Increasing the presence of these compounds inside the berry cell, beyond the quality aspect, could be a new challenge for the production of functional wine or grape juice as a marketing tool. Regulating vine water deficit is considered a tool for increasing flavonoid content and improving winegrape quality (*8*, *9*), and it has recently been seen how flavonoid biosynthesis gene expression is modified by water deficits (*10*). An increase of gene expression of the phenylpropanoid metabolism has been reported following postharvest grape dehydration (*11*, *12*) and a significant increase in *trans*-resveratrol has been found in Amarone wine (*13*). Postharvest water loss significantly affects primary metabolism (shift from aerobic to anaerobic respiration) and secondary metabolisms (synthesis of volatile compounds and polyphenols) depending on the rate and amount of water loss (*14*−*17*). Although genetic difference and maturity stage affect the rate of water loss during postharvest, management of postharvest grape dehydration depends on three environmental factors: temperature, relative humidity, and air flow. Temperature plays the main role because it affects not only the rate of mass transfer (water evaporation) but also the main metabolism. In Cesanese winegrapes an increase in flavonols, epicatechin, and *trans*-resveratrol has been observed during cluster partial dehydration (20% weight loss) at 20 °C compared to 10 °C treatment while anthocyanins diminished (*17*, *18*). Even in Montepulciano winegrape, a significantly higher decrease in anthocyanins at 20 °C than at 10 °C when the berries reached 40% weight was reported (*19*). No data exists on the temperature effect on gene expression of the phenylpropanoid pathway during postharvest dehydration, while, in the vineyard, Tarara et al. (*20*) showed how berry temperature affects anthocyanin composition and high

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temperatures reduce the endogenous ABA level, which in turn decreases the expression of the VvmybA1 protein (*21*), which controls the expression of the anthocyanin biosynthetic enzyme genes . Shade and sunlight conditions, through the control of myb protein products, can also affect the regulation of phenylpropanoid biosynthetic genes, as was found in Cabernet Sauvignon (*22*). In this study we used three temperatures—10, 20, or 30 °C—for postharvest dehydration in order to evaluate the effect of temperature on water loss and particularly on the polyphenol metabolism of Aleatico winegrape (*Vitis vinifera* L.), including the expression of 4 key genes of this metabolism and the activity of polyphenoloxidase as the oxidative enzyme during the dehydration process, which greatly affects the final color of the berry skin and, consequently, must color.

MATERIALS AND METHODS

Experimental Procedure and Quality Analyses. Grape clusters from an Aleatico grape vineyard grown around Viterbo without irrigation, in volcanic soil (lapillo) and on a hillside, were carefully harvested (sound berries and uniform size) early in the morning, when a soluble solids content (SSC) of 18 ± 1 °Brix was reached. After 1 h of transport under shaded conditions, clusters (arrival temperature at lab: 23 °C) were placed in perforated boxes (60 × 40 × 15 cm) in a single layer. For each test, two perforated boxes with 6 kg of clusters each were placed in a small metallic tunnel (45 × 45 × 100 cm) adapted with an exhaust fan with air-flow regulation. The small tunnels were placed in three thermohygrometric controlled rooms (12 m^3) at 10 (±1)°C, 20 (±1)°C, or 30 (±1)°C. Relative humidity was set at 45% (±5) in the three rooms and air flow at 1.5 m/s. The experiment lasted the time to reach 40% weight loss (wl) in our clusters. Sampling was performed at the beginning and then at 10, 20, 30 and 40% wl, and for molecular tests, biological samples were immediately frozen in liquid nitrogen and stored at −80 °C until analysis. Weight was carefully measured using a technical balance (Adam Equipment Co. Ltd., Milton Keynes, U.K.), and the SSC of the juice obtained from the berries was measured using a digital refractometer (Atago, Tokyo, Japan). Vapor pressure deficit (VPD) was calculated by using the automatic calculator by Autogrow System Ltd. (http://www.hydro.co.nz). The berry dehydration kinetics were obtained, as described in Barbanti et al. (*23*), by plotting the moisture content (M), measured at any sampling time, against the dehydration time (days). The initial moisture content (M0), which was used for *M* calculation, was obtained by following the AOAC method (*24*).

Biochemical and Chemical Analyses. A variety of polyphenols, including phenolic acids (caftaric and coutaric acid), anthocyanins (delphinidin 3-*O*-glucoside, cyanidin 3-*O*-glucoside, petunidin 3-*O*-glucoside, peonidin 3-*O*-glucoside, malvidin 3-*O*-glucoside, peonidin 3-(6-acetyl)-glucoside, malvidin 3-(6-acetyl)-glucoside, peonidin 3-(6-*p*-coumaroyl)-glucoside, malvidin 3-(6-*p*-coumaroyl)-glucoside), flavonols (quercetin 3-*O*-glucuronide, rutin, quercetin 3-*O*-glucoside, kaempferol 3-*O*-glucoside), stilbenes (*trans*-piceid, *trans*-resveratrol) and catechin were identified and quantified using high-performance liquid chromatography with photodiode array (PDA) detector which was hyphenated with a single quadrupole mass spectrometry equipped with an electrospray ion source (HPLC-PDA−ESI-MS). Frozen grape berries were powdered in liquid nitrogen and, after the seeds were removed, lyophilized. A weighed amount of the lyophilized sample in the range of 200−250 mg was quantitatively extracted with an 8:2 (v/v) methanol−ethanol mixture at room temperature for 2 h in a round-bottom flask. The extraction was repeated twice, and the collected supernatants were concentrated in a rotary evaporator with the heated water bath set at 35 °C. The residue was quantitatively recovered in 1 mL of 8:2 (v/v) methanol−water and used for HPLC-PDA−ESI-MS identification and quantification of polyphenols. The experiments were performed with a Shimadzu LCMS-2010 unit, comprising an SCL-10Avp system controller, two LC-10ADvp solvent delivery module pumps, an SPD-M10Avp UV−vis photodiode array detector, a single quadrupole 2010 mass analyzer equipped with an electrospray ESI, with nitrogen as the nebulizing and dehydration gas. The ESI-MS detection was performed in negative ionization mode for phenolic acids, flavonols, catechins, and stilbenes, and in positive ionization mode for anthocyanins. Optimized conditions were determined by flow injection analysis (FIA) of standard solutions of the analytes at three different concentrations, ranging from 0.1 to 50 mg/L. System control and data processing were carried out by Shimadzu LCMS solution software running on a personal computer. The compounds were separated using a Polaris C18A column (150 × 2 mm i.d., 5 μm particle size) (Varian Inc., Lake Forest, CA) in conjunction with a C18 (30 × 2 mm, 5 μm) guard cartridge column; the column temperature was 30° ± 1 °C. Separations were performed by a multistep gradient of increasing concentration of acetonitrile in an acetonitrile−water mixture containing 5% (v/v) formic acid, at a flow rate of 0.2 mL/min. Samples were introduced into the column using a semimicroinjection valve (model 8125; Rheodyne, Cotati, CA) with a 5 μL sample loop. Column effluent was passed through the photodiode array detector before being directed to the ESI interface and quadrupole mass spectrometer.

Polyphenol oxidase (PPO) activity was measured by applying the method described in Valero et al. (*25*) with modifications. 50 g of the peel and pulp tissues obtained from intact fresh grape berries was suspended in 25 mL (2:1) of 0.1 M Na-acetate buffer (pH 5) and homogenized in Ultraturrax. The homogenate was filtered under vacuum in a Buchner funnel using Whatman n.1 filter paper and than centrifuged at 4000*g* for 20 min. At the end of the centrifugation procedure, the supernatant was discarded and the recovered pellet was suspended in 20 mL of 0.1 M Na-acetate buffer (pH 5) containing 1.5% of Triton X-100 after the addition of 2 g of PVPP. The mixture was centrifuged at 13000*g* for 25 min. The supernatant obtained from the second centrifugation was the crude extract for the enzyme assays. All the operations were carried out following the cold chain at 4 °C. The assay mixture consisted of 1 mL of 0.1 M Na-acetate buffer (pH 5), 50 μL of 0.2 M 4-methylcatechol and 5 μL of crude extract. Enzyme activity was measured at 400 nm with a Lambda 3B UV−vis spectrophotometer (Perkin-Elmer Instruments Ltd., Seer Green, Beaconsfield, U.K.) reading against a blank consisting of 1 mL of 0.1 M Na-acetate buffer (pH 5) and 50 μL of 0.2 M 4-methylcatechol. The rise in absorbance observed over 2 min was due to the disappearance of the *o*-phenol and the corresponding *o*-quinone formation. The PPO activity was expressed as units per gram of dry weight. One unit represents the enzyme which is required for the liberation of 1 μmol/min of *o*-quinone at a standard temperature of 25 °C.

Cloning and Sequences of Candidate Genes. A PCR cloning strategy on target genes was applied to isolate nucleotide sequences corresponding to the following genes: PAL (phenylalanine ammonia lyase), DFR (dihydroflavonol reductase), CHI (chalcone isomerase), STS (stilbene synthase), elF1-α (elongation factor), and SAND family protein (SAND). The sense and antisense primers were designed on the basis of the cDNA of all gene sequences of grape berry present in GenBank. elEF1-α and SAND genes were used as housekeeping genes to provide a relative transcription level. Amplified products were purified using the Wizard SV Kit and cloned using the pGEM-T Easy Vector System, following the manufacturer's instructions (Invitrogen). Plasmids derived from cloning were verified by restriction digestion with *Eco*RI and later sequenced. Sequencing was performed on ABI 310 equipment using BigDye Sequencing Chemistry at the Tuscia University sequencing facilities. The putative coding sequences obtained were analyzed with BLASTn software (*26*) against nonredundant (nr) sequence database (version 2.2.10 and 2.2.14) and VitisGenomeAnalysis. The sequences obtained were used to design specific primers to be used in real time RT-PCR experiments.

DNA and RNA Extraction and Gene Expression Analyses. DNA was extracted by leaf tissues following the previously described protocol (*27*). Total RNA was extracted from grape berry pericarp tissues powdered under nitrogen following the protocols described by Reid et al. (*28*). RNA concentration and purity were determined before and after DNAase I digestion with a spectrophotometer (absorbance ratio 260 nm/280 nm), and 1.2% agarose gel run to visualize RNA integrity. For each grape berry peel sample, polyadenylated RNAs (mRNAs) were purified from 1 μg of total RNA using an RNeasy kit (Qiagen) and used as template for the synthesis of first strand cDNA. Reverse transcriptions (RT) were carried out using Ready-To-Go RT-PCR beads (Amersham Biosciences, Italy) according to the manufacturer's instructions. Forty units of RNAguard RNase Inhibitor (Amersham Biosciences, Italy) was added to the RT reactions. Quantitative real time RT-PCR analyses were carried out using the LightCycler system (Roche, Italy). Serial dilutions of a reference cDNA obtained from control samples were used as standard for the construction of the standard calibration curves. Amplification and

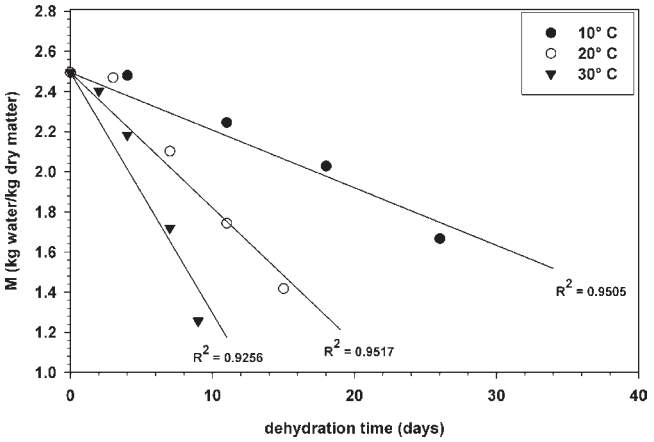

Figure 1. Dehydration kinetics of Aleatico grape clusters at 10, 20, and 30 °C. Measurements were taken at harvest and when the cluster weight loss reached 10, 20, 30, and 40%. Moisture content (M = kg of H_2O/kg of dry matter at any time) was calculated starting from initial moisture (M0).

detection were performed using a Sensi mix kit (Quantace, Italy), including SYBR green fluorescent dye (Roche, Italy). The qRT-PCR tests were carried out on three biological replicates using the LightCycler platform (Roche Molecular Diagnostics). The reactions were carried out in a total volume of 20 μL, with 1.5 mM MgCl2 and 10 mM of each primer, 1.5 μL of Taq polymerase, 4 μL of buffer plus dNTP, 1 μL of SYBR green dye and 5 μL of cDNA (corresponding to 3 ng of cDNA). These cDNA template amounts were adjusted for each gene according to the sensitivity threshold and the linearity range of the respective standard calibration curves. Aliquots from each RNA sample were used with all primer sets in each experiment. Reactions were run using the following thermal parameter: starting denaturation of 10 min, a total of 40 cycles of 10 s at 95 °C, 12 s at 59 °C, 12 s at 72 °C, dissociation curve for each amplicon were than analyzed to verify the specificity of each amplification reaction. The relative quantification was obtained with the ΔCP method (*29*), which normalizes the CP values of target genes using the correlated reference CP value, and using the PCR efficiency value (E) obtained from the respective standard calibration curve.

Statistical Analyses. Specific polyphenols and anthocyanins were determined in triplicate. Analysis of variance was performed by ANOVA and significance evaluated per $p = 0.05$. Mean values were compared by Tukey's test ($\alpha = 0.05$) and significant difference graphically attributed by letters. Calculations were performed by Minitab 15 (Minitab, Inc., USA).

For gene expression, ANOVA was performed for normalized transcript level. Differences were accepted as statistically significant when $p < 0.05$. Post hoc comparison was performed using the Student−Newman−Keuls test (SNK).

RESULTS AND DISCUSSION

Under constant RH and air flow, 40% weight loss was reached after 8, 14, and 26 days at 30, 20, and 10 °C, respectively. The plot of mass loss (kg of water/kg of dry matter) against time of dehydration provided straight decreasing linearity with high R^2 of 0.92 ($y = -0.1199x + 2.4943$), 0.95 ($y = -0.0674x + 2.4943$) and 0.95 ($y = -0.0287x + 2.4943$) at 30, 20, and 10 °C, respectively (**Figure 1**). The reason for the different dehydration time is mainly due to the vapor pressure deficit (VPD), 0.64, 1.28, and 2.33 kPa for 10, 20, and 30 °C treatment, respectively. The rate of weight loss (%/day) was 5, 2.9, and 1.5 at 30, 20, and 10 °C, respectively. It is to be emphasized that, even with the same percentage of water loss, the berry texture was different. As observed in a previous paper by Bellincontro et al. (*17*), the berry dehydrated at 10 °C appears with more uniform shape than the one of berry dehydrated at 20 °C, and this is due to a slower and regular transfer of water from the berry core to the berry surface during dehydration at 10 °C, while at 20 °C and even more at

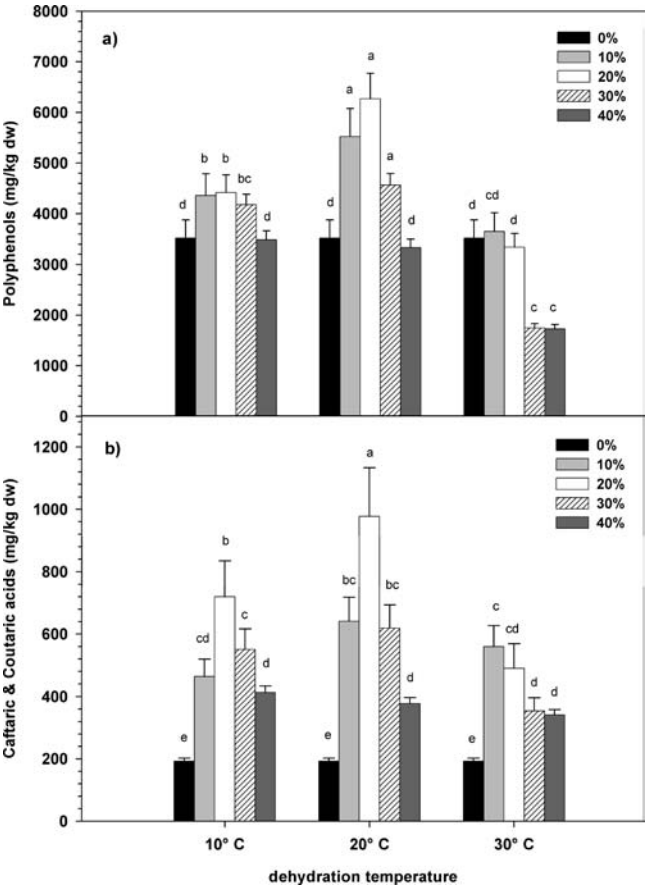

Figure 2. (**a**) Total polyphenol content (mg/kg dry weight) determined by HPLC in Aleatico grape berries dehydrated at 10, 20, or 30 °C. Measurements were taken at harvest and when the cluster weight loss reached 10, 20, 30, and 40%. Data are the mean of three HPLC analyses from three different sets of berries. Vertical bars indicate SD. Values with different letters were significantly different ($p < 0.05$). (**b**) Caftaric and coutaric acid content (mg/kg dry weight) determined by HPLC in Aleatico grape berries dehydrated at 10, 20, or 30 °C. Measurements were taken at harvest and when the cluster weight loss reached 10, 20, 30, and 40%. Data are the mean of three HPLC analyses from three different sets of berries. Vertical bars indicate SD. Values with different letters were significantly different ($p < 0.05$).

30 °C this process is faster and not uniform (*30*). The increase in sugars was similar for all three samples, reaching 27.5 °Brix for the 10 °C sample and 28.5 °Brix for the other two samples. During the concentration in sugars, a significant difference was found at 10 and 20% wl between 30 and 10 °C samples, the latter with a lower rate of increase. This different behavior is likely due to the different dehydration time: 10% wl was reached after 2 and 4 days respectively at 30 and 10 °C, and 20% wl after 4 and 11 days. It is supposable that, during this longer period of time, the respiration rate, even at a lower rate, continues and consumes substrate. This event has been seen during long-term dehydration under uncontrolled conditions and has been attributed to the fermentation process occurring in the berry cells (*31*). At 30 °C the fast water evaporation causes rapid texture degradation, initially enzymatically dependent linked with cell wall enzymes (*32*) and, subsequently, physically dependent, which could permit the increase in refractometric materials, sugars and mineral ions, thus the increase in the SSC. Better texture maintenance at 10 °C and 20% of weight loss compared to the sample kept at 20 °C was observed (*17*). As a consequence of water stress after harvest, we observed a large increase in the total content of the analyzed

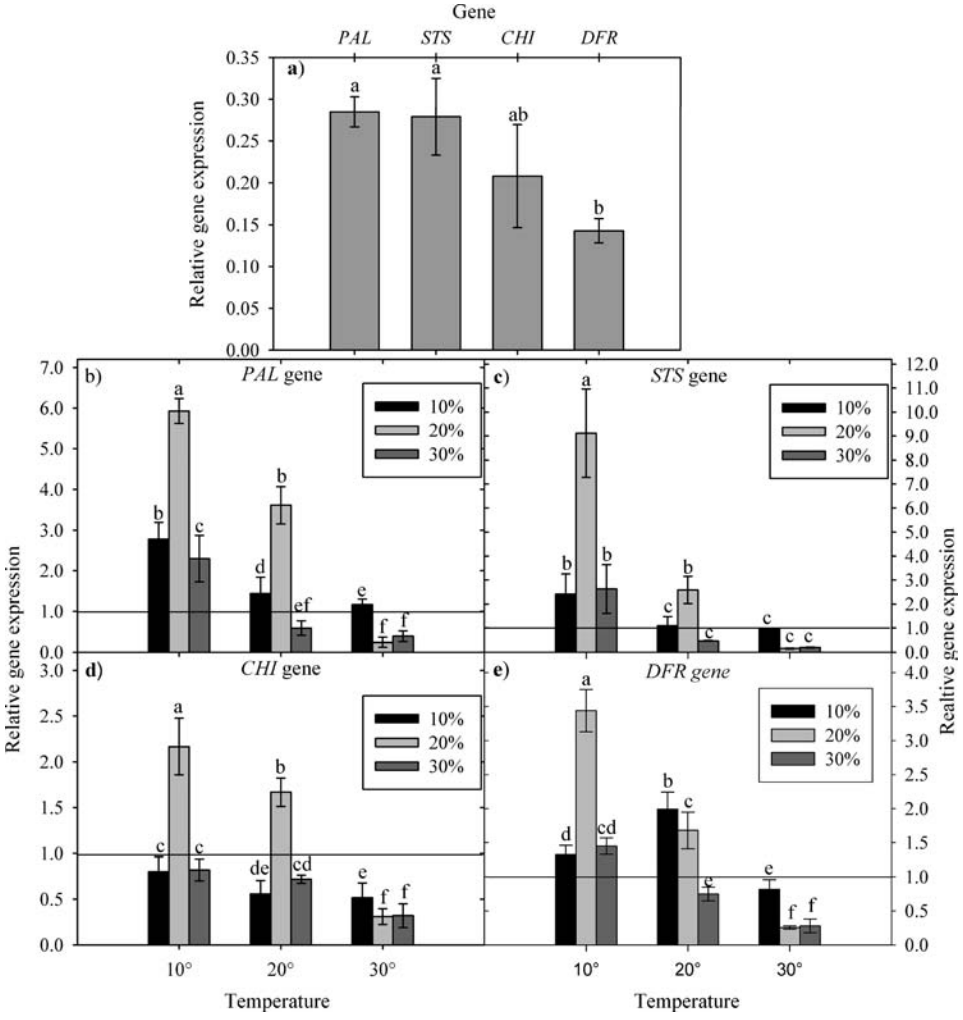

Figure 3. Relative expression of analyzed genes as detected on pericarp of grape barriers. (**a**) Relative gene expression before the berries exposure at the different treatments. (**b**, **c**, **d**, **e**) Relative expression of PAL, STS, CHI and DFR gene, respectively, in exposed berry samples along the period of treatments. Histograms represent the average of three biological replicate samples that were independently detected. The data were normalized on the basis of housekeeping gene (eIF1-α) and referred to the control at time zero. Bars represent the ±SD. Averages were separated on the basis of temperature treatment, using the SNK test.

polyphenols, confirming that berry dehydration induces general phenylpropanoid metabolism, which generates precursors for many different categories of phenolic compounds (*11*). Total polyphenols increased slightly at 10 °C with 10% wl and to a significantly greater extent at 20 °C, reaching the maximum (almost double) at 20% wl; polyphenols decreased at 30 °C (**Figure 2a**). More recently, Antelmi et al. (*33*) using Aleatico grapes coming from the same vineyards as those used in our experiment, but well irrigated (thus clusters were subjected only to postharvest water stress), showed that the weight loss peak was at 30% after 11 days at 20 °C. In our study, double water stress occurred: the first in the field and the second after harvest. This double water stress caused a more rapid response, after 6 days, and at lower weight loss, 20%. It is presumable that the lower cell turgor at harvest due to lack of irrigation induced physical changes in cell membrane structure linked to lipoxygenase and fatty acid content (*34*) as well as on surface wax (*35*), making the cells more sensitive to further water loss. The phenolic acids caftaric and coutaric acids, which significantly change during dehydration (*36*), increased in all the samples until 20% wl at 10 °C, and even more so at 20 °C, to decline later on, while at 30 °C the increase occurred at 10% wl and then declined (**Figure 2b**). This increase confirmed what was observed in Aleatico by Frangipane et al. (*37*) using rising temperatures during postharvest dehydration, and it has recently been seen in Pedro Ximenez grapes dehydrated at 40 and 50 °C also (*38*), where the increase was higher than expected as a result of water evaporation from grapes. The expression of PAL gene, coding phenylalanine ammonia lyase enzyme, which is active at the beginning of the phenolic acids pathway, was upregulated in the berries exposed to temperatures of 10° and 20 °C, over the dehydratation of 10% of weight loss (**Figure 3b**), while in the unexposed berries, at the beginning of treatments, the gene resulted less transcript than the elF1-α, the housekeeping gene (**Figure 3a**). However these observations are partially in sound with that from the metabolic accumulation products. Transcript abundance of PAL gene did not well correlate with metabolite abundance, and the peak of accumulated phenolic acids has been found in berries exposed at 20 °C at 20% wl (**Figure 2b**), while the peak transcript abundance has been found in berries exposed at 10 °C at 20% wl (**Figure 3b**). The effect of wl on the regulation of PAL gene expression is already reported (*12*), but any relationship is reported with the temperature of exposure. In citrus, low temperature played an inductive role of the expression of genes coding for the enzymes regulating the phenylpropanoid pathways (*39*); therefore diverse regulatory processes, independent of transcript abundance, could

have occurred in the metabolic adjustments of berries at different temperature and dehydration pressure conditions. The mechanism underlying the berry sensing system and the subsequent signal transduction generated in the cells could be differently affected by the exposure at diverse temperatures, which in turn have determined different exposure time to reach the same degree of dehydration. The above hypothesis is confirmed by other experiences from studies on both transcript and metabolic profiling of whole pathways regulated by the contemporary action of diverse environmental stress factors (*40*). The same behavior of PAL gene expression was likewise observed for the other analyzed phenylpropanoid pathway genes. DFR gene resulted upregulated in the berries exposed to temperatures of 10 and 20 °C, over the dehydratation of 10% of weight loss, with the peak transcript abundance at 10 °C and 20% wl (**Figure 3e**). In contrast the amount of CHI transcript detected in the exposed berries (**Figure 3d**) was lower with respect to the unexposed ones (**Figure 3a**), regardless the dehydration temperature at 10% wl, therefore this gene resulted downregulated. Along the prolonged exposure of berries to both temperatures of 10° and 20 °C, the level of gene expression increased over time during the dehydration of berries up to the weight loss of 20%, as may be deduced by the highest amount of transcripts detected (**Figures 3b** and **3e**). The berries exposed to a temperature of 30 °C always showed a reduced accumulation of all gene transcripts, and in most of cases the amount of accumulated transcripts of each gene was less than the amount determined before the treatments. Following the increase in phenolic acids, stilbene compounds such as *trans*-resveratrol and *trans*-piceid increased 4-fold at 10% wl at 20 °C, from 30 mg/kg dw up to 100, and remained at this level until the end of the experiment (**Figure 4a**). At 10 and 30 °C the increase was significant but lower (around 60 mg/kg dw) while, at 10 °C, it occurred at 20% wl and then remained stable; at 30 °C the peak was reached at 10% wl and then rapidly declined. Flavonols such as quercetin 3-*O*-glucoside and kaempferol 3-*O*-glucoside increased significantly only at 20 °C at 20% wl, while in the other samples it remained stable (10 °C) or significantly declined (30 °C) (**Figure 4b**). Stilbenes are known as protective phenolic compounds that are activated under stress conditions (*41*). A strong induction of resveratrol due to water stress during postharvest grape dehydration for the production of Amarone has already been observed (*13*), and recently eight tags corresponding to STS genes (TC52790, TC52907, TC53668, TC59572, TC60946, NP1227286) were induced by withering, suggesting a large stilbene production (*11*). Stilbene and chalcone pathways share the same precursor (4-coumaroyl-CoA and malonyl-CoA), and, for this reason, if one pathway is overexpressed, the other pathway can be downexpressed. Our results seem to confirm this assumption: at 10 °C stilbenes increased slightly, while flavonols remained stable; at 20 °C stilbenes increased significantly immediately (10% wl) and remained high, while flavonols increased later and then declined; at 30 °C, which is the strongest stress condition, stilbenes increased immediately (10% wl) and then declined, while flavonols decreased. Catechins immediately increased 4-fold at 20 °C and 10% wl to decline rapidly, while at 10 °C the increase was double at 20% wl as well as at 30 °C, to decline later on (**Figure 4c**). Castellarin et al. (*10*) have shown that the synthesis of proanthocyanidins is predominant at the preveraison stage, and after veraison the flavonoid pathway shifts to flavonols and anthocyanins. Our rapid increase in catechins at 20 °C, similar to that of stilbenes, might indicate a strong stress condition in which stilbene plays a defense role, while catechin might act like antioxidant compounds. An increase in catechin and epicatechin has been found in grape on the vine subjected to water deficit (*9*) and in Pedro Ximenez grape postharvest dried at

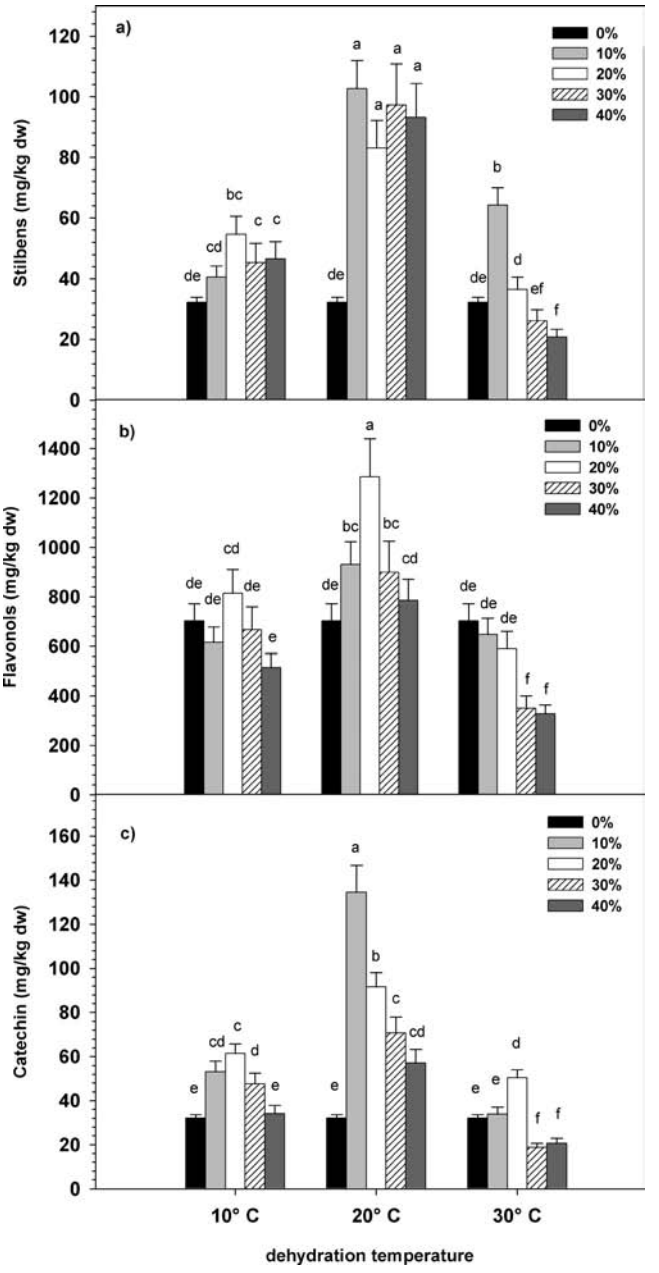

Figure 4. (**a**) Stilbene (*trans*-resveratrol and *trans*-piceid) content (mg/kg dry weight) of Aleatico grape berries dehydrated at 10, 20, and 30 °C. Measurements were taken at harvest and when the cluster weight loss reached 10, 20, 30, and 40%. Data are the mean of three HPLC analyses from three different sets of berries. Vertical bars indicate SD. Values with different letters were significantly different ($p < 0.05$). (**b**) Flavonol content (mg/kg dry weight) of Aleatico grape berries dehydrated at 10, 20, and 30 °C. Measurements were taken at harvest and when the cluster weight loss reached 10, 20, 30, and 40%. Vertical bars indicate SD. Values with different letters were significantly different ($p < 0.05$). (**c**) Catechin content (mg/kg dry weight) of Aleatico grape berries dehydrated at 10, 20, and 30 °C. Measurements were taken at harvest and when the cluster weight loss reached 10, 20, 30, and 40%. Data are the mean of three HPLC analyses from three different sets of berries. Vertical bars indicate SD. Values with different letters were significantly different ($p < 0.05$).

40 °C, but not at 50 °C (*38*). As suggested by Serratosa et al. (*38*), the postharvest dehydration process is a balance between synthesis and oxidation processes. We add that this response depends on temperature and amount of water loss. At a high dehydration temperature (i.e., 30 °C) the biosynthetic process occurs rapidly,

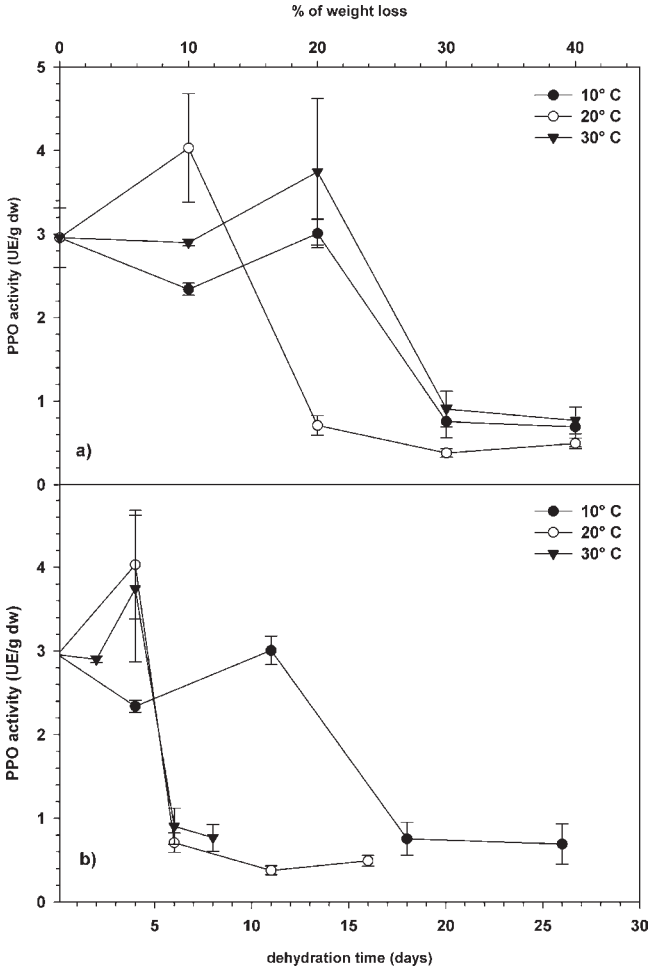

Figure 5. (**a**) PPO activity of Aleatico grape clusters dehydrated at 10, 20, and 30 °C and plotted against sampling time (0, 10, 20, 30, and 40% of wl). Data are the mean of 3 enzyme activities from three different sets of berries. Vertical bars indicate SD. (**b**) PPO activity of Aleatico grape clusters dehydrated at 10, 20, and 30 °C and plotted against dehydration time (days). Data are the mean of 3 enzyme activities from three different sets of berries. Vertical bars indicate SD.

especially for defense compounds such as stilbenes, but immediately declines because of the physical alteration of cells and rapid enzymatic and nonenzymatic oxidation, which lead to cell death. Upregulated genes related to perception and response to stimuli for cell rescue, defense and virulence were found in Raboso grape dehydrated mainly due to rapid water loss more than to amount of water loss (*12*). Indeed, this latter factor affects the reaction ability of tissue to a greater degree, and this is the reason for the decline in most of the phenolic compounds analyzed at greater weight loss and confirms what was observed by Rizzini et al. (*12*) comparing 30% weight loss to 10%. Moreover, in avocado an increase in phenylalanine ammonia lyase (PAL), chalcone synthase (CHS) and flavanone 3 hydroxylase (F3H) activities and a consequent increase in procyanidins such as epicatechin following ethylene treatment have been observed (*42*). Rizzini et al. (*12*) recently observed that 1-aminocyclopropane-1-carboxylate (ACC) oxidase, responsible for the conversion of ACC to ethylene, appeared upregulated in all postharvest dehydrated grape berries, suggesting that ethylene might be involved in the response of grape berry to postharvest water stress. When the amount of water loss is high (i.e., above 30% weight loss), whatever temperature is used, physical alteration occurs, leading to cell death, with a similar effect as when high temperature is

Table 1. Total Content of Anthocyanins, Mono-, Acetyl- and Cumaroyl-Glucoside Anthocyanins Determined by HPLC (mg/kg dry weight) in Aleatico Grape Berries Dehydrated at 10, 20, or 30°C until 10, 20, 30, and 40% Weight Loss Was Reached[a]

temp, °C	0%	10%	20%	30%	40%
		Monoglucoside			
	1755.4 def				
10		2290.2 g	1860.0 ef	1732.6 def	1690.0 cde
20		2458.8 g	2459.8 g	1976.4 fg	1496 bcd
30		1687.0 cde	1423.6 bc	608.4 a	619.7 a
		Acetyl Glucoside			
	258.6 de				
10		306.7 ef	253.6 cde	273.1 def	211.8 bc
20		375.3 g	371.5 g	254.8 de	191.7 bc
30		248.1 cd	289.1 ef	100.8 a	106.5 a
		Coumaroyl Glucoside			
	550.4 cd				
10		586.1 d	653.9 d	860.0 e	550.5 cd
20		882.4 e	1024.2 e	647.6 d	333.4 a
30		423.8 bc	527.1	287.8 a	281.3 a
		Total Anthocyanins			
	2564.5 d				
10		3183.1 f	2767.5 e	2865.8 e	2452.4 cd
20		3716.8 g	3855.5 g	2878.9 e	2021.3 b
30		2359.9 c	2451.0 cd	997.1 a	1007.6 a

[a] Data are the means of three HPLC analyses from three different sets of berries. Values with different letters inside the single group of compounds were significantly different ($p < 0.05$).

used. Thus, we can summarize that a lower temperature such as 10 °C reduces and delays the stress effect, as indicated by the lower phenolic synthesis; a higher temperature (30 °C) emphasizes the rapid response of some known stress compounds such as resveratrol or phenolic acids; 20 °C is the temperature which provides the complete and fully expressed response to stress because this temperature is known as the optimal condition for enzyme activity. Indeed, analysis of PPO revealed significant activity at 20 °C, corresponding to the increase in catechin (10% wl), while at 10 and 30 °C, the rise was delayed to 20% weight loss with a similar level of activity for the sample at 30 °C, and significantly lower for the 10 °C one (**Figure 5a**); in contrast, catechin concentration was much lower at 30 °C than at 20 °C. In terms of dehydration time, samples at 20 and 30 °C showed the peak on the same day (4), while the sample at 10 °C showed the peak on the 11th day (**Figure 5b**). The same behavior was observed for catechin, where the peak was concomitant on day 4 for samples at 20 and 30 °C and on the 11th day for the sample at 10 °C (data not shown). Since polyphenols are stored in vacuoles and PPO is located in cytoplasm, they can only come into contact if the tonoplast is altered and catechin can be released into the cytoplasm. Catechin is a flavan-3-ol monomer involved in the formation of tannins, which are extremely toxic compounds for the cell due to their protein denaturant activity (*43*). For this reason catechin is an optimal substrate for PPO activity and the high activity of PPO is due to the need for catechin detoxification. Similar behavior was observed in postharvest dehydrated grapes from an irrigated Aleatico vineyard, but the response at 30 °C was much lower (*33*). The further decline in PPO with the progressive weight loss is due to the reduction in catechin content, as well as to the disruption of chloroplast since PPO is localized on the thylakoids of chloroplasts.

Total anthocyanins increased significantly of 20 and 31% at 10% wl, respectively, at 10 and 20 °C while it decreased at 30 °C (**Table 1**). At 20 °C, the content further increased at 20% wl to decrease later on but still was significantly higher than the initial value. Even at 10 °C the content remained higher than the initial value while it dramatically decreased at 30 °C at 40% wl. An increase in anthocyanins during postharvest ethylene treatment of winegrapes has been reported (*44*), and a strict correlation between ethylene and anthocyanins has been observed in winegrapes after field treatment with ethephon (*45*). As we mentioned earlier, an involvement of ethylene in the berry response to postharvest water stress has been postulated (*12*). Thus, the observed rapid increase in anthocyanins could be attributed to stress response ethylene, which is more evident at 20 °C, less at 10 °C and no response at 30 °C. Further on, in the case of lipid peroxidation, it has been seen in grape berry extracts that catechin can also work for the regeneration of the highly efficient antioxidant malvidin 3-glucoside and, to a lesser extent, peonidin 3-glucoside (*46*). In addition, a stimulation of the gene coding for dihydroflavonol reductase (DFR), an enzyme involved in the biosynthesis of some anthocyanins, has been observed in in vitro culture of cells of grape berry of the cultivar Gamay Red after treatments with sucrose, glucose and fructose (*47*). The decrease of anthocyanin concentration at elevated temperatures might result from both a decrease of synthesis and an increase of degradation, as was previously suggested by Shaked-Sachray et al. (*48*) and demonstrated by Mori et al. (*49*). Strong oxidation, which involves enzymes such as PPO, but also peroxidase, could be one of the causes. Grapevine peroxidases are glycoproteins of high thermal stability capable of accepting a wide range of natural compounds as substrates, including anthocyanins (*50*). They are located in cell walls and vacuoles. Although peroxidase is a constitutive enzyme in grapevines, its levels are strongly modulated during plant cell development and in response to both biotic and abiotic environmental factors. A higher temperature such as 30 °C, which causes rapid water loss and thus cell wall and plasma membrane degradation, may induce peroxidase activity which, in turn, attacks oxidizable substrates such as anthocyanins. All the other anthocyanin fractions behave like total anthocyanins. Monoglucoside content peaked at 10% wl at 10 and 20 °C, to decrease successively at slightly lower level than the initial value at 40% wl; at 30 °C a loss of about 66% occurred at the last sampling. Same behavior was for acetyl glucoside content while coumaroyl glucoside increased significantly (34%) at 10 °C until 30% wl to decline greatly to the initial level at 40% wl. At 20 °C, 20% wl, coumaroyl content reached a value 50% higher than the initial level, and then declined significantly at lower level than the initial one. At 30 °C berries diminished and, at the end of test, showed the lowest value. Computing the percentage of acylated anthocyanins over the total, samples at 10 °C maintained the same value (31%) for the entire dehydration period, while samples at 20 °C, after an initial increase from 31 to 36%, decreased to 26%; finally, at 30 °C an increase from 31 to 38% was observed due to the greatest decrease in total anthocyanins in the step between 20 and 30% wl. Indeed, the loss was 60, 58, 66, 58% for total anthocyanins, cumaroyl, acetyl, and monoglucoside, respectively. The ratio between esterified anthocyanins and the total has been considered variety-dependent, but it appears that postharvest stress conditions can change this ratio, as has been observed in the field (*19*).

In conclusion, the postharvest dehydration response of Aleatico grape in terms of phenolic compounds is strongly dependent on the dehydration temperature, and the amount of weight loss. 20 °C appears to be the temperature which expresses the most complete response in terms of the increase in compounds of antioxidant interest (stilbenes, catechins, quercetin) but only until 20% of weight loss is reached. 10 °C is the best temperature for maintaining the amount of anthocyanins until the time the berries reach a weight loss of 40%. 30 °C is a detrimental temperature for postharvest dehydration of Aleatico grape.

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