Postharvest Wine Grape Dehydration: ethanol dissipation from grape and biochemical changes

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Short title: **Ethanol dissipation in Postharvest Grape Dehydration**

Abstract

BACKGROUND: During postharvest dehydration, grapes are subject to metabolic changes including ethanol anabolism and catabolism. These changes affect the quality of the final product and ethanol production is a key step. Ethanol dissipation has never been measured during postharvest wine grape dehydration thus the present study aimed: i) to monitor ethanol dissipation; ii) to study chemical-biochemical changes in berries during dehydration.

RESULTS: Ethanol dissipation from Raboso grapes was measured, under controlled postharvest dehydration up to 36 % weight loss (w.l.). Moreover, the activity of enzymes involved in the anaerobic metabolism of grapes was studied. Ethanol dissipation was highly correlated with grape weight loss (R^2 = 0.989). Alcohol dehydrogenase (ADH) activity, responsible for the reduction of ethanol to acetaldehyde, declined significantly with the w.l. Similarly, pyruvate decarboxylase (PDC) and lactate dehydrogenase (LDH) reduced their activity. High lipoxygenase activity (LOX) was measured at 27 % w.l., while polyphenol oxidation (PPO) was constant and declined in the last sampling.

CONCLUSION: Ethanol dissipation during postharvest dehydration allows for reducing anaerobic metabolism and promotes oxidative metabolism. The sensor used can be a useful commercial tool to monitor the berry metabolism.

Keywords: Postharvest dehydration, Ethanol dissipation, Enzymatic activity, Ethanol sensor

INTRODUCTION

As ripening progresses, O_2 concentration approaches zero in grape seeded cultivars and is correlated to cell death (CD) across the mesocarp[1]. O_2 increases towards the central axis corresponding to the presence of air spaces which are connected to the pedicel where lenticels are located which are important for berry O_2 uptake as a function of temperature; when lenticels are blocked, a hypoxia condition starts in grape berries, as well as ethanol accumulation and CD[2–4]. Xiao et al. (2018)[5] demonstrated that in non-irrigated vines, berry O_2 decreased, while both CD and ethanol concentration increased. Water stress is the typical stress occurring during postharvest wine grape dehydration, which induced great changes in terms of metabolism[6,7]. Water stress switches from aerobic to anaerobic metabolism (in the presence of oxygen or not), depending on the rate of water loss, and increases ADH activity and ethanol formation[8]. Malate has been proposed to act as a carbon source in aerobic ethanol fermentation to provide adenosine triphosphate (ATP) during high rates of fruit metabolism[9]. This induction is likely due to sugar excess, which increases the rate of glycolysis and pyruvate levels[10,11]. It has been observed that during postharvest wine grape dehydration and when temperature, relative humidity (RH), and air flow are controlled, aerobic fermentation takes place in grape berries, starting at 20 % w.l. with an increase in ADH activity[4,8,12–14]. Recently, it has been observed that malic acid can be considered as a marker of this metabolic shift because its content initially decreases and then increases due to a concentration effect[15]. Plants produce a wide spectrum of biogenic volatile organic compounds (BVOCs) in different tissues to communicate with other plants and organisms. For instance, plants emit BVOCs to attract insects and to respond to biotic and abiotic stresses[16]. The volatile terpenes family is certainly one of the most abundant BVOCs but also methanol and acetaldehyde are important constitutive and induced volatiles of many plants[17,18]. If methanol is the consequence of cell wall pectin degradation and cell stress condition, acetaldehyde emission from plants correlates with root flooding and with xylem sap ethanol concentration[19,20]. In these conditions, ethanol formed under anoxic conditions in roots is transported to leaves by the transpiration stream, where it is oxidized to acetaldehyde by ADH. However, only a small portion of acetaldehyde is emitted while the bulk is further metabolized by aldehyde dehydrogenase (ALDH) to acetate and acetyl-CoA[16]. In plants, the dissipation of these volatiles is easy owing to the presence of a great number of stomata and lenticels. On the other hand, grape berries are known as a conservative organ which becomes remarkably insensitive to changes in plant water status and acquires considerable tolerance to water stress because of its very low number of stomata or non-stomata activity[21,22]. As previously described, during ripening or in water stress conditions, ethanol accumulates in the berry flesh[1,5]. During postharvest dehydration of grape bunches, rachis drying is very fast, and about 5 - 7 % of the initial w.l. of the bunch is due to rachis w.l.[23]. When rachis w.l. is completed, the connection between pedicel, receptacle and berry becomes loose, thus favouring gas release from the berry. Indeed, it is known that the most of gas exchange in fruit covered by wax occurs through stem scar[24,25]. The presence of epicuticular wax increases the insensitivity to water loss and its removal accelerates berry softening and degradation, including oxidation and browning phenomena[26]. It has been recently demonstrated that the storage of grape berries treated with 1-methylcyclopropene (1-MCP) or stored under high oxygen or carbon dioxide atmosphere condition leads to an increase in alcohol along the storage period[27].

Based on our previous research[8,12,14,28,29], the hypothesis of this experimental work has been that ethanol accumulates in ripening berries and that it is metabolized but also dissipated during postharvest dehydration. As such, the monitoring of atmosphere ethanol dissipation during the postharvest grape dehydration process at controlled temperature, RH, and air flow, has been carried out by using a commercial ethanol sensor managed by an Arduino-type console created specifically. Chemical and biochemical changes in the berry were also studied in order to better clarify berry metabolism in postharvest dehydration conditions.

MATERIALS AND METHODS

Raw materials and experimental design

Raboso grapes (*Vitis vinifera* L.) were provided by Sensi Vigne & Vini (Lamporecchio, Italy). Grapes were hand harvested in the Veneto Region when their sugar content was about 190 - 200 g L^{-1} and immediately shipped to the University of Pisa (1 h away).

Twenty-four whole bunches were washed individually with chlorinated water (5 - 10 % Cl) and then superficially dried; bunches were accurately separated and placed into two perforated plastic crates for the dehydration process. A commercial fan (SN2003, Senelux, Liverpool, United Kingdom) was used to produce an air speed of 1.5 m s^{-1} during the day (12 hours) through the crates. The rest of the time the fan was off.

The operating conditions were as follows: temperature (T) = 21 ± 1 °C, RH = 65 – 75 %. Air speed, T and RH (relative humidity) were constantly monitored by a multiparameter sensor (Lutron LM-8000, Coopersburg, Pennsylvania, USA).

Each bunch was marked and individually monitored during the dehydration process, which lasted up to 36 % w.l. The sampling was performed approximately every week.

Chemical analysis

Sixty collected berries from 3 different bunches (20 berries each rep) at each sampling time were homogenized in a blender (LM435810 Blendforce 2, Moulinex, Écully, France). The bunches were different from the ones used to compute w.l. The juice (one for each replicate) was first centrifuged (6869 *g* for 5 min at 20 °C) and filtered (paper filter 0.82 µm) and then analysed by a calibrated Fourier transform infrared WineScan™ FT 120 (Foss Analytics, Hillerod, Denmark) to determine the following oenological parameters: sugars (g L^{-1} hexoses), pH, ethanol (g L^{-1}), titratable acidity (g L^{-1} tartaric acid), volatile acidity (g L^{-1} acetic acid), malic acid (g L^{-1}), tartaric acid (g L^{-1}), Yeast Assimilable Nitrogen (YAN) (mg L^{-1}), total anthocyanins (mg L^{-1} malvidin), and total polyphenols (mg L^{-1} gallic acid). For each juice sample (three in total) three readings have been taken. The accuracy of the WineScan™ was verified by destructive analyses performed as previously reported[30].

Ethanol sensor measurement

The ethanol sensor consisted in a commercial MQ-3 sensor (metal oxide semiconductor gas sensor) (Hanwei Electronics, Zhengzhou, China) and we created an Arduino PC card platform with remote control. The two MQ-3 sensors consisted of a ceramic tube (Al_2O_3) coated with a thin film of SnO_2. MQ-3 is a conductometric-type gas sensor where the gas absorbed on the surface of the tin dioxide (SnO_2) material changes its resistance at a temperature of around 350°C (Figure 1a). This change in resistance *R*s together with the external resistance R_L and the dc supply V_L activates a voltage divider circuit that produces an output voltage V_{RL}, correlated with the gas concentration[31]. MQ-3 sensor is sold for ethanol gas analysis, but it can read also other alcohols such as methanol, hexanol. The readings were expressed by the sensors in mg L^{-1} of alcohol in the jar headspace.

To test the firm accuracy of the sensor, different ethanol gas concentrations were released into a glass jar environment. Specifically, liquid ethanol (96 % of purity, PanRea, Applichem, Perlabo sas, CT, Italy) from 0.1 up to 5 mL, was poured on filter paper in an open Petri dish (to allow the gradual evaporation of ethanol) placed inside an empty glass jar (1 L volume) with the ethanol sensor, and immediately closed.

During postharvest dehydration, the ethanol produced by grape bunches was measured by placing two bunches (total weight about 200 g) taken from the perforated plastic crates inside a glass jar (volume 1 L) equipped with the MQ-3 sensor, and accurately capped (Figure 1b). The bunches were kept inside the jars for 2 hours at the same ambient conditions of

dehydration test. Ethanol measurements were taken weekly throughout the dehydration process; each time two jars were used (for each jar the same total weight of bunches was used).

Enzymatic activity

Total soluble proteins were extracted by resuspending 1 g of frozen grape berry powder (10 collected berries from 3 different bunches (10 berries each bunch) at each sampling time were used) in 5 mL of extraction buffer (100 mM potassium phosphate buffer pH 7.8), 1 mM sodium EDTA (pH 7), 5% (w/v) PVPP supplemented with 2 mM DTT, 1 mM PMSF, 0.2 % Triton X-100. The homogenate was centrifuged at 18000 *g* for 10 min at 4 °C and the supernatant was used for enzymatic activities. The protein content was measured for each individual enzyme studied[32].

PPO (EC.1.10.3.1) was determined as previously reported[33]. First, 2.5 g of berries were homogenized in 5 mL of 100 mM sodium phosphate buffer pH 6.4 containing 0.125 g PVPP. Crude enzyme extract (100 µL) was incubated with a buffered substrate (500 mM catechol in 100 mM sodium phosphate buffer pH 6.4) in a final volume of 1.5 mL and monitored by measuring the increase in absorbance at 398 nm. The specific activity for molar change in catechol was expressed as mol mg^{-1} protein.

LOX (EC 1.13.11.12) was quantified following the method described by Petriccione et al. (2018)[33]. The enzyme was extracted by resuspending 1 g of frozen berry tissue powder with 3 mL of extraction buffer (50 mM potassium phosphate buffer pH 7.8, 1 mM sodium-EDTA pH 7, 2% PVPP). The reaction mixture consisted of 0.093 M sodium phosphate buffer pH 6, 0.17 mM linoleic acid sodium salt, and 50 µL of crude enzyme extract in a final volume of 1.5 mL. LOX activity was detected spectrophotometrically by recording the formation of hydroperoxides and the resulting increase in absorbance at 234 nm. LOX activity was expressed as the specific rate of molar change of hydroperoxides in nmol mg^{-1} protein.

ADH (EC 1.1.1.1) was measured as described by Costantini et al. (2006)[8]. Five grams of powder, obtained from frozen and ground grape berries, was suspended in 10 mL (1:2) of 1 M Tris-HCl buffer (pH 7.4) containing 5 mM DTT, 1 mM EDTA, and 1% w/v PVP Polyclar-AT. The homogenate was centrifuged at 15000 *g* for 10 min at 4 °C and the supernatant was used for the assay. The assay mixture consisted of 0.6 mL of crude extract in 2.7 mL of incubation substrate containing 0.1 M L-glycine (pH 9.6), 0.33 M NAD^+, and 0.2 mL of 0.3 M water solution of ethanol. Enzyme activity was measured at 20 °C by reading the rise in absorbance at 340 nm due to the NADH formation following ethanol oxidation. The ADH content was expressed as mmol mg^{-1} protein.

PDC (EC 4.1.1.1) was measured as described by Lara et al. (2011)[34]. Extraction from grape berry tissue was performed by using 85 mM MES (pH 6.5), 25 mM NaCl, 1 mM $MgCl_2$ and the assay of crude extract by adding 2mM DTT, 2 mM thiamine pyrophosphate 0.15 mM NADH, 50 mM oxamate, 3 U of ADH, and 10 mM pyruvate, where oxamate acts as an inhibitor of LDH activity. Crude extracts were pre-incubated for 30 min in the reaction media in the absence of pyruvate, NADH and ADH. The PDC content was expressed as mol mg^{-1} protein.

LDH (EC 1.1.1.27) was quantified following the method described by Lara et al. (2011)[34]. Extraction was performed as PDC. For the assay, 50 mM NaPi (pH 7.5), 10 mM pyruvate, 0.2 mM NADH; and 1 mM methylpyrazole, which inhibits PDC activity, were used. The LDH content was expressed as mol mg^{-1} protein.

All the enzymes were calculated on protein content which was computed on a dry weight (d.w.) basis.

Statistical analysis

Data were first analysed using the Shapiro-Wilk and Bartlett test to verify normality and homogeneity of variances. After checking these pre-requisites, data were compared by one-way ANOVA and Tukey's honestly significant difference (HSD)

test with $p < 0.05$ for multiple comparison by using CoStat Software (Version 6.451, CoHort Software, Pacific Grove, CA, USA).

RESULTS AND DISCUSSIONS

Enochemical features and weight loss

Weight loss in Raboso grapes showed a linear increase and reached 36 % in 30 days (Figure 2a), confirming a constant dehydration ambient condition. As expected, the increase of sugar content on a w.l. basis showed a regression straight line trend, with an $R^2 = 0.975$ due to the concentration effect by w.l. (Figure 2b).

As far as the other analytical parameters are concerned, pH decreased during the dehydration process while titratable acidity significantly increased, again due to the concentration effect of tartaric acid (Table 1). The decrease of pH is in relation with the increase of acidity and it is unusual because it is a common behaviour that pH does not decrease with the increase of titratable acidity during postharvest wine grape dehydration[35]. This event occurs overall during grape dehydration at low temperature while in our case the rapidity of the process at room temperature, hindered the salification.

Malic acid content confirms its well-known dynamic behaviour in grape berry under stress conditions[36]. It decreased progressively to 18 % w.l. due to the induced water stress, and then it rose again reaching a value higher than the initial one owing to concentration (Table 1). This pattern was already observed in Amarone grapes under postharvest dehydration[15].

Volatile acidity was almost undetected at the first sampling (0.04 g L^{-1}). Its level increased with the progress of the dehydration process reaching 0.45 g L^{-1} at 18 % w.l.; later the level remained constant with a slight increase at the last sampling (Table 1). The increase of volatile acidity is a well-known mechanism induced during post-harvest dehydration due to induced water stress and the consequent shift aerobic/anaerobic fermentation and cell death[12,28]. Overall, this results in an increase of volatile acidity. This is an important aspect because during winemaking, especially in sugar-rich must, volatile acidity increases by about 1 g L-1 and at the end its concentration might be too high. YAN increased significantly, and progressively, due to the concentration effect[8,23]. Finally, total anthocyanins did not change for most of the dehydration process; at 27 % w.l., the concentration increased at about 30 % (Table 1). Total polyphenols rose at 12 % w.l., likely as a water stress response, then decreased due to oxidation, and rose again at about 30 % at the end of dehydration, due to the concentration effect[37]. This behaviour confirmed what was observed by Rizzini et al. (2009)[38] in Raboso grapes subjected to postharvest dehydration.

Ethanol dissipation

Following the water loss trend, a significant ethanol increase was measured in the atmosphere around the bunches; ethanol in the headspace was highly correlated with w.l. ($R^2 = 0.989$) (Figure 3).

For the first time, this pattern has been measured in an atmosphere surrounding grape bunches during wine grape postharvest dehydration, and confirms what was postulated on ethanol formation inside the berry during postharvest w.l.[1,8,12,14].

As already mentioned, grape berries accumulate ethanol during ripening which cannot be released in the environment because the berry skin is almost completely waterproof. During postharvest w.l., the grape rachis dries rapidly, representing about 5-7 % of total bunch w.l.[23]. The loss of physical connection between the berry, the dried rachis and the receptacle caused by water loss enables the release of internal gases into the environment, favouring ethanol dissipation. The loss of physical connection between pedicel and berry has previously been demonstrated with the penetration of *Alternaria* spp through the berry-pedicel interface (beneath the stem cap)[39]; thus, it is plausible that, in the same way, gas exchange could occur through this interface. Postharvest treatment with ethanol on table grapes produced elevated levels of acetaldehyde and ethanol in the berry which decreased during shelf-life in 3 weeks[39], without any water loss. Thus, ethanol dissipates

outside from the berry or is metabolized. To confirm this assumption, ethanol measured by WineScan™ FT 120 in the berry juice decreased from 0.14 to 0.05 g L^{-1} at 36 % w.l. (Table 1). As aforementioned, QM-3 sensor is not specific for ethanol even though is the alcohol mostly read, but it could also read methanol and other minor alcohols. Even though methanol is read by the sensor, its concentration in the headspace compared to the one of ethanol is minimal at our knowledge during grape dehydration. At WineScan™ reading of grape juice the values were negative, meaning that the concentration was neglectable.

Biochemical changes

During postharvest dehydration, protein content increased from 0.115 mg g^{-1} d.w. up to 0.175 (12 % w.l.), then declined to 0.134 (18 – 27 % w.l.) to rise again in the end, to 0.400. The protein content (and the relative enzymes activity reported below) was computed on a d.w. basis, thus not affected by the concentration effect. This protein pattern was reported also by Costantini et al. (2006)[8] in Malvasia grape postharvest dehydration under controlled condition, to indicate intense anabolism and catabolism. ADH (ethanol to acetaldehyde) activity decreased progressively (Figure 4a).

Also, PDC (Figure 4b) and LDH (Figure 4c) decreased progressively with the increase of w.l., at a lower rate than ADH. At the last sampling, the activities greatly decreased. Our results of ADH activity are in contrast with what was observed by Costantini et al. (2006)[8] and Cirilli et al. (2012)[14] who reported a progressive increase of ADH activity. Costantini et al. (2006)[8] showed an initial increase of ethanol content in grape tissue and then a decrease. Zenoni et al. (2016)[7] reported the activation of lactate dehydrogenase and the down-regulation of alcohol dehydrogenase, while Cirilli et al. (2012)[14] observed a different ADH expression in grapes in postharvest dehydration condition, depending on the temperature of dehydration. Regarding these response discrepancies, Zenoni et al. (2016)[7] concluded, "However, our data were based on berries subjected to slower and more prolonged dehydration compared to these other studies, and inconsistencies in the expression of genes related to anaerobic respiration may therefore reflect such differences". A recent paper on wine grape postharvest dehydration by Zenoni et al. (2020)[6] reported that grape dehydration kinetics should be carefully controlled by setting appropriate environmental parameters, thus supporting the transcriptomic and metabolic changes that lead to the development of desirable quality traits; different dehydration regimes affect the final quality of the berry, as shown by molecular changes that occur during postharvest dehydration and their relation to different environmental parameters.

In conclusion, the data obtained suggest that the ripening berry is initially in an anoxia condition [1], therefore the activity of PDC, LDH and also ADH (in one direction and in the other) is very intense. Subsequently, a hypoxia condition occurs. As such, ADH activity (ethanol to acetaldehyde) is initially high because of the anoxia condition, then it declines because ethanol dissipates (hypoxia), and because it is metabolized to acetic acid. This metabolism is promoted by the oxidation caused by air entrance due to the lack of physical connection between the berry and the receptacle caused by dehydration; consistently, the volatile acidity (acetic acid) increased significantly at 18 % w.l. (Table 1). Ethanol loss from the berry tissue was reported also by Costantini et al. (2006)[8]. Thus, the initial anoxia condition of the berry cell tends to shift to hypoxia. The activity of PDC and LDH decreased at a lower rate than ADH because, in a condition of high ethanol content, LDH pathway is favoured to maintain the acidity of the medium. At 36 % w.l., at our knowledge, cell death could occur and this the reason of the drop of the enzymatic activity. Over-expression of lactate dehydrogenase and downregulation of ADH have been observed during postharvest dehydration of wine grapes[7,38,40].

The results of the PPO and LOX analyses were conditioned by the penetration of air into the berry because of rachis drying, which contributes to the shift from anoxia to hypoxia. PPO activity was constant up to 27 % w.l. and then decreased as expected (Figure 4d). LOX activity was constant up to 18 % w.l. and significantly increased at 27 % w.l., while it decreased at the end (Figure 4e). PPO showed a constant high activity because the high affinity of PPO for oxygen is well-

known in grapes[41]; this high activity of PPO could explain the reduction of polyphenols at 18 and 27 % w.l. (Table 1). The increase of LOX at 27 % w.l. confirms the presence of air inside the cell. This increase at a certain percentage of water loss during postharvest dehydration of the grape berry has been previously reported[8,12,28,33,42].

CONCLUSIONS

The process of postharvest water loss in grape berries involves several steps, including oxidative and osmotic stress response, anaerobic respiration, defence response, cell wall metabolism, and carbohydrate metabolism. For the first time, we demonstrated ethanol dissipation during postharvest dehydration of wine grapes. Together with ethanol release from the berry, important metabolic changes occur involving a reduction of ADH activity and, to a lesser extent, PDC and LDH. Moreover, the entrance of air into the berry favours the oxidation process due to the high activity of LOX and oxidation of polyphenols caused by PPO. This result confirms the anaerobic metabolism in the berry cell during postharvest dehydration due not to the presence of bacteria on berry surface and this applied ethanol sensor can be useful, commercially speaking, to monitor the berry metabolism evolution.

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DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 1. Enochemical features of grapes at different percentages of weight loss (%).

Parameters	0 %	7 %	12 %	18 %	27 %	36 %
pH	3.56 ± 0.02 a	3.58 ± 0.04 a	3.43 ± 0.03 b	3.41 ± 0.01 b	3.28 ± 0.02 c	3.33 ± 0.03 c
Ethanol (g L^{-1})	0.14 ± 0.02 a	0.13 ± 0.02 a	0.07± 0.02 b	0.07 ± 0.01 b	0.06 ± 0.01 bc	0.05 ± 0.01 c
Titratable acidity (g L^{-1} tartaric acid)	6.31 ± 0.12 d	6.66 ± 0.16 c	6.82 ± 0.11 b	7.04 ± 0.14 a	6.96 ± 0.18 ab	7.14 ± 0.13 a
Volatile acidity (g L^{-1} acetic acid)	0.04 ± 0.02 d	0.08 ± 0.01 cd	0.12 ± 0.04 c	0.41 ± 0.04 b	0.45 ± 0.04 b	0.53 ± 0.03 a
Malic acid (g L^{-1})	2.36 ± 0.06 b	2.35 ± 0.08 b	2.21 ± 0.09 c	2.19 ± 0.10 c	2.39 ± 0.03 b	2.64 ± 0.07 a
Tartaric acid (g L^{-1})	4.44 ± 0.14 c	4.49 ± 0.13 c	4.64 ± 0.11 c	4.53 ± 0.11 b	5.12 ± 0.14 b	5.53 ± 0.15 a
YAN (mg L^{-1})	154 ± 7 d	164 ± 9 d	187 ± 8 c	195 ± 11 c	231 ± 7 b	278 ± 12 a
Total anthocyanins (mg L^{-1} malvidin)	685 ± 19 bc	697 ±11 b	669 ± 22 d	677 ± 13 cd	842 ± 16 a	865 ± 13 a
Total polyphenols (mg L^{-1} gallic acid)	1843 ± 35 d	1884 ± 31 d	2209 ± 26 b	2089 ± 34 c	2071 ± 21 c	2493 ± 13 a

Data are the mean (± SD) of 3 berry set (20 berries from 1 bunch, 3 different bunches used) and are expressed on a fresh weight basis.

In the same row, different letters indicate significant differences among values ($p < 0.05$).

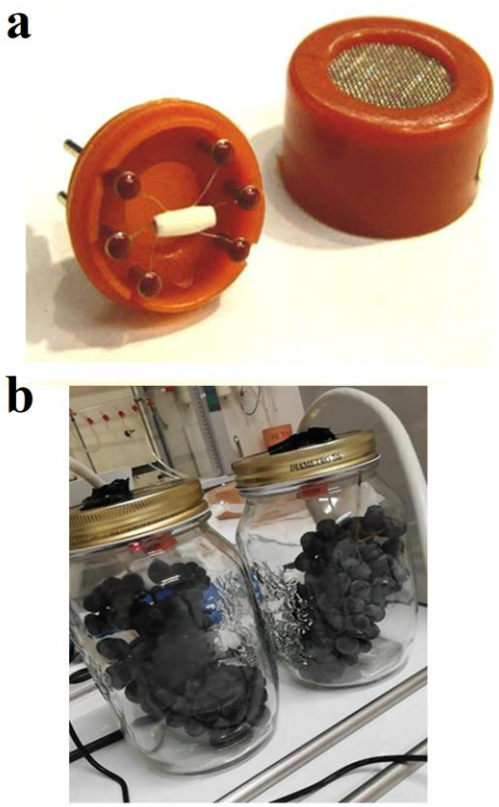

Figure 1. (**a**) MQ-3 sensor used for ethanol detection in air; (**b**) Sensor-equipped jars and bunches during ethanol measurement.

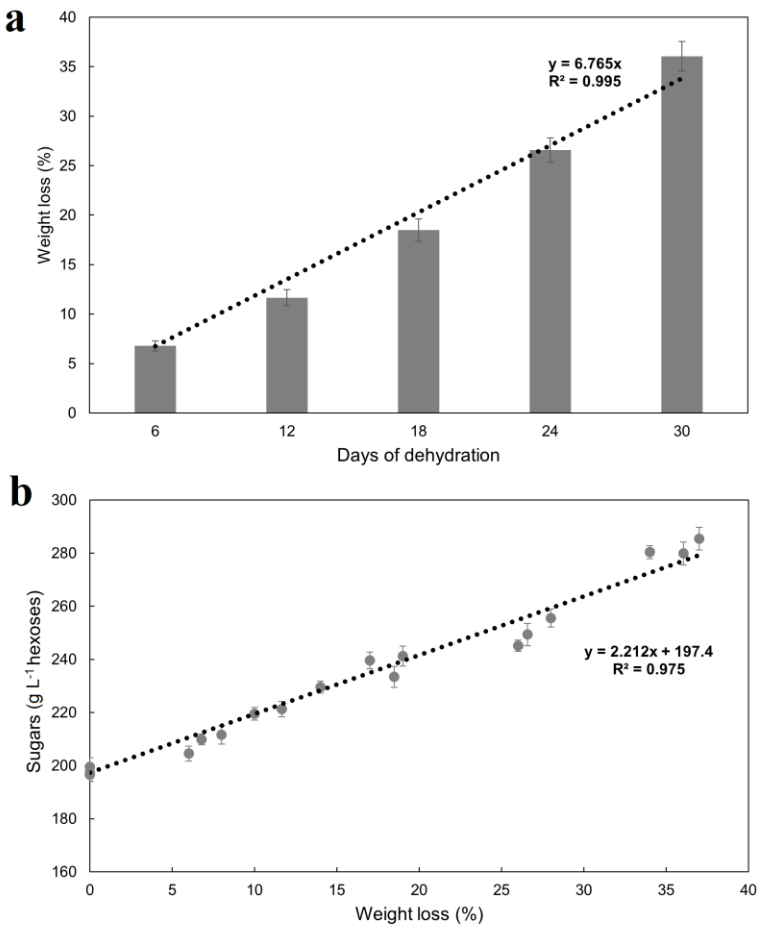

Figure 2. (a) Evolution of weight loss (%) as a function of dehydration days. Data are the mean (± SD) of 10 grape bunches, 5 each plastic crates; **(b)** Evolution of sugar content (g L^{-1} hexoses) as a function of weight loss (%). Data are the mean (± SD) of 3 berry sets from 3 different grape bunches.

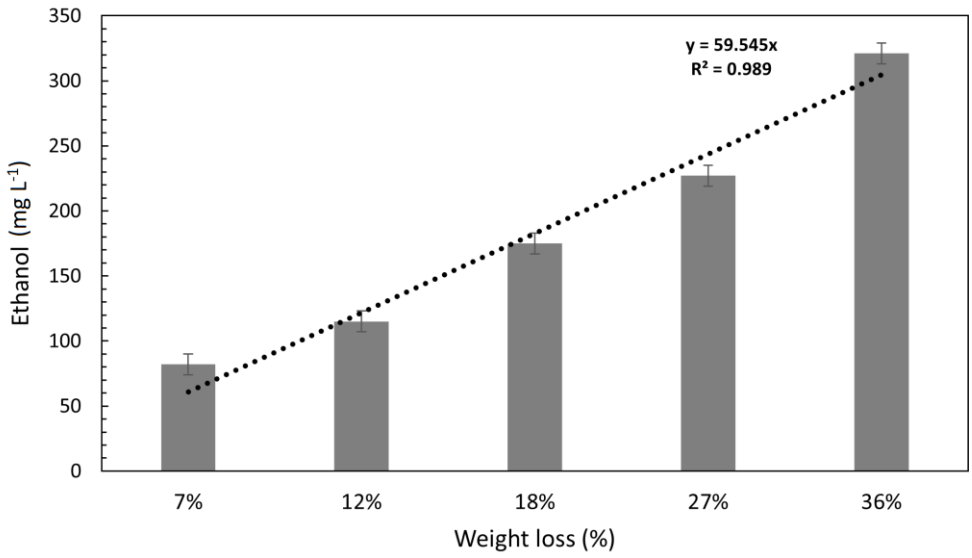

Figure 3. Ethanol concentration (mg L^{-1}) recorded by the sensors as a function of weight loss (%). Data are the mean (± SD) of the readings from two sensors, each one in a single jar with grape bunches.

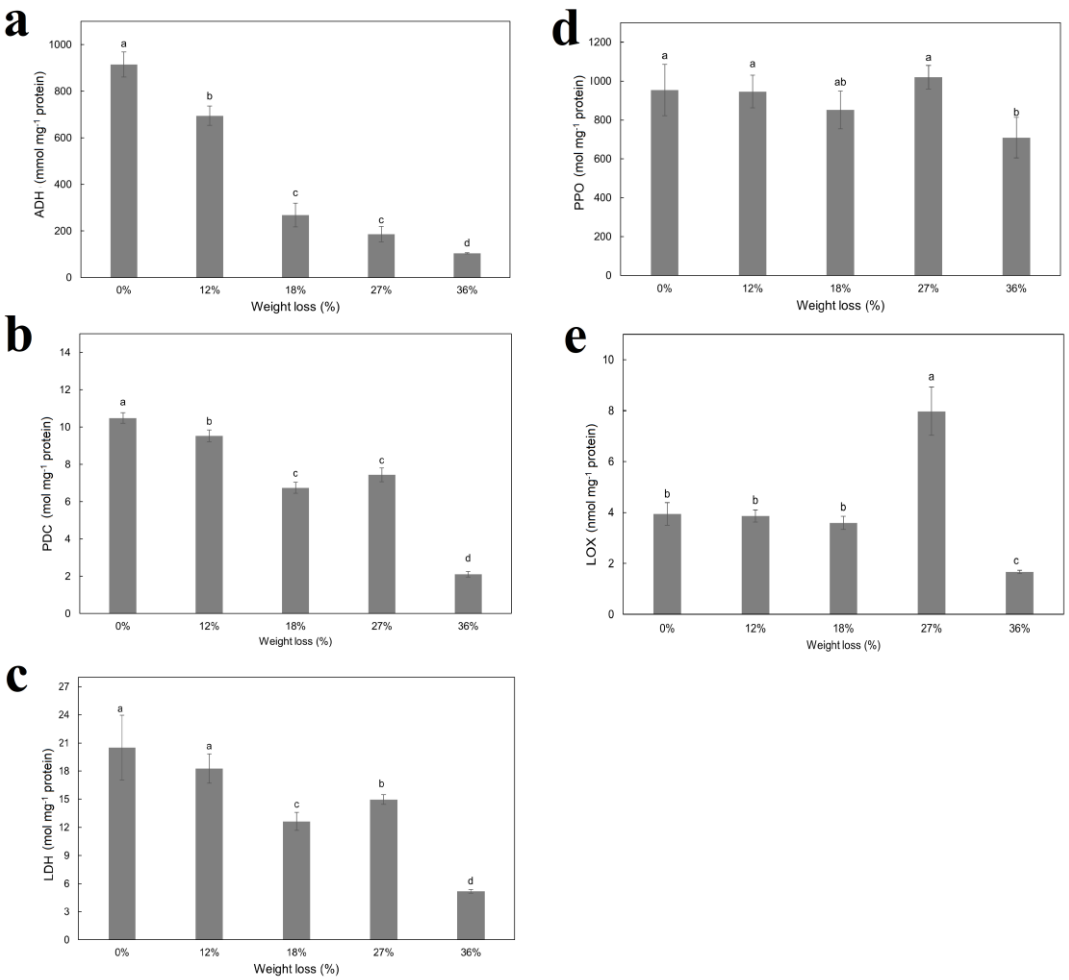

Figure 4. Enzyme activity [mg of protein (on dry weight basis)] of grape berry at different percentage of weight loss; **(a)** ADH; (**b)** PDC; **(c)** LDH; **(d**) PPO; **(e)** LOX. Data are the mean (± SD) of 3 berry sets from different grape bunches. Different letters in the same figure differ significantly for $p < 0.05$.