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Changes of Ochratoxin A in Grapes Inoculated with *Aspergillus carbonarius* and Subjected to Chamber-Drying under Controlled Conditions

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The production pattern for ochratoxin A in grapes inoculated with *Aspergillus carbonarius* and changes in its concentration during raisining of Merlot, Syrah, Tempranillo, and Cabernet Sauvignon red grapes and Pedro Ximenez white grape were studied. Grapes were chamber-dried under controlled temperature and humidity conditions, with and without dipping pretreatments in alkaline emulsions of olive oil or ethyl oleate. Based on the results for the grapes that developed the fungus (Merlot and Pedro Ximenez), a temperature of 50 °C in the absence of dipping stopped ochratoxin A production and even degraded part of the toxin already formed. Both dipping pretreatments facilitated removal of the toxin and led to its virtually complete disappearance. However, dipping in the ethyl oleate emulsion caused substantial changes in the sensory characters of the musts obtained from the raisins, so it should be avoided to ensure the expected quality in the sweet wines elaborated from them.

KEYWORDS: Grape drying; *Aspergillus carbonarius*; ochratoxin A

INTRODUCTION

Ochratoxin A is a mycotoxin essentially produced by fungal species of the genera *Aspergillus* and *Penicillium* with nephrotoxic, teratogenic, and immunosuppressive effects. Also, it has been included in Group 2B (potentially carcinogenic to humans) by the Agency for Research on Cancer (*1*). This toxin is present in foods such as cereals, coffee, dried fruits, cocoa, beer, and wine (*2*−*4*). Wine has been deemed the second most important source of ochratoxin A in the human daily intake, which has led to the establishment of increasingly low allowed limits for the toxin in wines, grapes, and raisins. Current European legislation is based on EC Regulation 1881/2006 of Dec 19, 2006, which set a maximum tolerated level of 2.0 μg/L for grapes harvested since 2005.

Ochratoxin present in wine comes from the vine plant, where the toxin-producing fungus occurs since the earliest stages of berry growth. This makes early detection of some fungi such as *Aspergillus carbonarius* crucial because it is known to be one of the greatest contributors to the presence of the toxin in grapes (*5*), especially under a warm climate such as that typical of the Mediterranean region (*6*). Specifically, grapes may be contaminated as early as the veraison period (*7*, *8*), their subsequent development and ochratoxin A production being influenced by different factors, particularly those related to climatic conditions during ripening (*5*). Temperature is an important influential factor, peaking for example the ochratoxin A production at 15−20 °C in *A. carbonarius* and at higher temperatures (20−25 °C) in *Aspergillus niger* (*9*). Also, growth of toxin-producing fungi is influenced by water activity in the grapes, the optimum value for which ranges from 0.930 to 0.987. As a result, different grape varieties exhibit also different sensitivity to the action of such fungi (*10*). Additional factors potentially affecting unwanted fungal development include the fungicide concentration and application time, as well as grape storage conditions.

During winemaking ochratoxin A passes from grapes into wine to an extent dependent on the above-described factors and also on the particular techniques used. Therefore, as expected, studies on wines from the same location but different vintages have shown ochratoxin A levels to be related to the climatic conditions of the year (*11*, *12*). Also, ochratoxin A production has been found to depend on wine type, red wines usually containing the toxin in increased amounts relative to rosé and white wines (*13*). This is mainly as a result of must from red grapes being macerated together with their skins. In addition, some researchers have found a relationship between ochratoxin A levels and production location in countries such as Italy and Greece and found that grapes growing in southern places of Europe are especially prone to develop the fungus and produce greater amounts of the toxin (*11*, *14*), particularly in red wines (*15*). Natural sweet wines obtained from raisins have been found to contain higher ochratoxin A concentrations than dry wines (*11*, *14*, *16*), as a result of the production process of the former including sun-drying of the grapes in order to raise their sugar contents, which increases the risk of contamination by ochratoxin A producing fungi.

Ochratoxin A is a moderately stable molecule at high temperatures and can withstand some thermal treatments used to

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Table 1. Physical and Chemical Parameters of Berry and Skin in the Studied Grapes and Homogeneous Groups

		Pedro Ximenez	Merlot	Cabernet Sauvignon	Tempranillo	Syrah
berry	average weight (g)	2.81 ± 0.025	1.27 ± 0.028 b	1.05 ± 0.020 a	2.26 ± 0.026 d	1.66 ± 0.064 c
	surface area (mm^2)	7.35 ± 0.115	4.54 ± 0.070 b	3.77 ± 0.066 a	6.51 ± 0.212 c	4.56 ± 0.228 b
	volume (mm^3)	1.76 ± 0.187	0.920 ± 0.020 a	0.700 ± 0.017 a	1.57 ± 0.077 b	0.927 ± 0.073 a
	surface area/volume	3.99 ± 0.150	4.92 ± 0.030 b	5.40 ± 0.028 c	4.13 ± 0.072 a	4.93 ± 0.125 b
	reducing sugar (g/L)	195 ± 1.00	224 ± 0.577 c	216 ± 1.52 b	190 ± 0.577 a	231 ± 0.577 d
	acidity (mequiv/L)	28.0 ± 1.74	40.3 ± 1.30 a	76.4 ± 1.45 b	160 ± 1.48 d	90.4 ± 0.900 c
skin	% weight	15.1 ± 0.585	25.8 ± 0.071 b	33.2 ± 0.542 c	18.7 ± 1.00 a	24.8 ± 0.793 b
	% water	80.5 ± 0.775	71.6 ± 0.963 a	75.5 ± 0.448 b	74.7 ± 0.756 b	75.7 ± 0.353 b
	reducing sugar (mg/g)	146 ± 1.58	186 ± 1.78 c	148 ± 1.05 b	130 ± 1.01 a	151 ± 0.868 b
	acidity (mequiv/g)	0.032 ± 0.000	0.051 ± 0.000 a	0.065 ± 0.000 b	0.064 ± 0.001 b	0.072 ± 0.000 c
	total polyphenol index (au)	2.47 ± 0.264	14.9 ± 0.142 c	11.5 ± 0.102 b	21.2 ± 0.156 d	10.8 ± 0.023 a
	A420 (au)	0.010 ± 0.000	0.168 ± 0.000 d	0.112 ± 0.000 b	0.153 ± 0.000 c	0.108 ± 0.000 a
	A520 (au)	0	0.305 ± 0.000 d	0.201 ± 0.000 b	0.268 ± 0.000 c	0.189 ± 0.000 a

process foods (e.g., roasting, baking) (*17*). However, its thermal stability is dependent on various factors such as the product moisture content and treatment duration (*18*). The toxin can be removed by using various physical and chemical treatments (*19*). Recently, some microorganisms and enzymes have proved to be quite effective for this purpose (*20*). Also, extracts from winemaking yeasts (*Saccharomyces*) have been successfully used to detoxify ochratoxin A contaminated grape musts (*21*).

In this work, we studied the ochratoxin A production patterns in grapes inoculated with *A. carbonarius* and fungal development in terms of ochratoxin A concentration during chamber-drying under controlled temperature and moisture conditions of grapes subjected to dipping or no pretreatments. Although the raisins obtained are usually destined to the production of sweet wines, the study of the changes of ochratoxin A in the wines was not the objective of this work.

MATERIALS AND METHODS

Grape Samples. The grapes used were of four different red varieties (Merlot, Syrah, Tempranillo, and Cabernet Sauvignon) and one white variety (Pedro Ximenez). All were harvested from the same plot in the Montilla−Moriles Designation of Origin (southern Spain) and all at their typical technological degree of ripening for standard winemaking. The agrochemical treatments used were identical for all varieties.

Grapes were carefully skinned by hand and their skins dried with filter paper prior to analysis. An aliquot of berries of each variety and their skin masses was heated to dryness (constant weight) at 100 °C in a stove in order to calculate the proportion of their respective dry solids. From these data were calculated the moisture content in the grapes and the percent water in the skins.

All analytical determinations were performed on triplicate musts from pressed berries or skin extracts obtained by using a hydroalcoholic mixture containing 12% (v/v) ethanol. Also, all measurements on berries and skins were made in triplicate using three samples of 100 berries per variety.

Inoculation with *A. carbonarius*. The study was conducted on strain A941 of *A. carbonarius* (fungus producer of ochratoxin A), which was previously isolated from grapes by Esteban et al. (*9*) for the Culture Collection of Veterinary Faculty of Barcelona (CCFVB) in Spain. The fungus was stored at −80 °C until use. The growth medium was agar-YPD (1% yeast extract, 2% peptone, 1% glucose, and 2% agar), and the incubation time 48 h at 28 °C. As sporulation medium was used corn meal agar 1.7%, yeast extract 0.1%, glucose 0.2%, and agar 2%, that was incubated at 28 °C during 48 h. The spores developed in corn meal agar were collected in sterile water. All grapes were inoculated by using a paintbrush to apply a sterile solution of fungal spores in distilled water on the total surface of each berry. After inoculation, the grapes were kept at 25 °C for 216 h.

Drying Experiments for Raisin Production. In a first experiment, a 6 kg batch of grapes inoculated of each variety was evenly distributed in a single layer and chamber-dried at a controlled air temperature of 50 °C and air moisture of 20%. In a second experiment, the grapes were previously dipped in a volume of 5 L of appropriate solutions and then dried in the same way and conditions as before. The treatment solutions and dipping times used were as follows: D0, without treatment; treatment D1 in which grapes were dipped in an alkaline emulsion containing 7% K_2CO_3 and 0.4% commercial olive oil at ambient temperature (22 °C) for 1 min; and treatment D2 in which grapes were dipped in an alkaline emulsion containing 2.5% K_2CO_3 and 2% ethyl oleate at ambient temperature for 10 s.

In both tests, grape samples were periodically collected to determine their weight loss. The chamber-drying was stopped when the reducing sugar content of the untreated grapes (D0) was about 330−350 g/L (measured in °Brix).

Raisins were crushed and pressed on an industry-like vertical press in the laboratory. The highest pressure reached in each pressing cycle was 300 bar, and each raisin batch was pressed in three cycles. The musts thus obtained were centrifuged at 3000 rpm prior to the different analyses.

Analytical Determinations. Reducing sugars were determined with an Atago Master manual refractomer that was calibrated over the °Brix range from 0 to 40 and by using the Luff−Schoorl method (*22*). Titratable acidity was determined by the European Community official method (*22*) and the colored fraction by using the absorbance at 420 nm as browning index and that at 520 nm as a measure of red color. The total polyphenol content was obtained with the Folin−Ciocalteau method (*22*).

Determination of Ochratoxin A. Ochratoxin A was determined with the procedure commercially available from R-Biopharm Rhône (Glasgow, Scotland). A volume of 10 mL of must was adjusted to pH 7.8 with 2 M NaOH, diluted with an identical volume of saline phosphate buffer at pH 7.3, and centrifuged at 1600 rpm for 10 min. Then, a volume of 20 mL of diluted sample was passed through an Ochraprep immunoaffinity column from R-Biopharm Rhône at a flow rate of 3 mL/min. The column was washed with 20 mL of buffer at pH 7.3 at a flow rate of 5 mL/min. Ochratoxin A was eluted with 1.5 mL of a 2:98 acetic acid/methanol mixture, followed by 1.5 mL of distilled water, both fractions being collected in a glass vial. An aliquot of 50 μL of eluate was injected into a Spectra-Physics Series P100 HPLC instrument equipped with a Perkin-Elmer Series 200 fluorescence detector.

The toxin was identified by comparison of its retention time with that for the R-Biopharm Rhône standard and by injecting the samples simultaneously with the standard. The quantification was made by using a calibration curve obtained with the same standard. The column used was a 250 mm × 4.6 mm i.d., 5 μm, Li-Chrospher 100 RP-18, with a 4 mm × 4 mm i.d. guard column of the same material (Merck, Darmstadt, Germany). The solvent used was a 99:99:2 acetonitrile/water/acetic acid mixture at a flow of 1 mL/min in the isocratic mode. The wavelengths used for fluorescence detection were λ_{exc} 333 nm and λ_{em} 470 nm.

Statistical Procedures. Multiple comparison procedures of means, variance analyses (ANOVA), and multivariate analyses (Principal Components) were performed on the replicated samples by using the Statgraphics Statistical Computer Package (Statistical Graphics Corp. v5.0).

RESULTS AND DISCUSSION

Characterization of Grapes. Pedro Ximenez white grapes and Merlot, Cabernet Sauvignon, Tempranillo, and Syrah red grapes were characterized from berry size, acidity, and sugar content related parameters. The results of the determinations are shown in **Table 1** together with those for some characteristics of grape

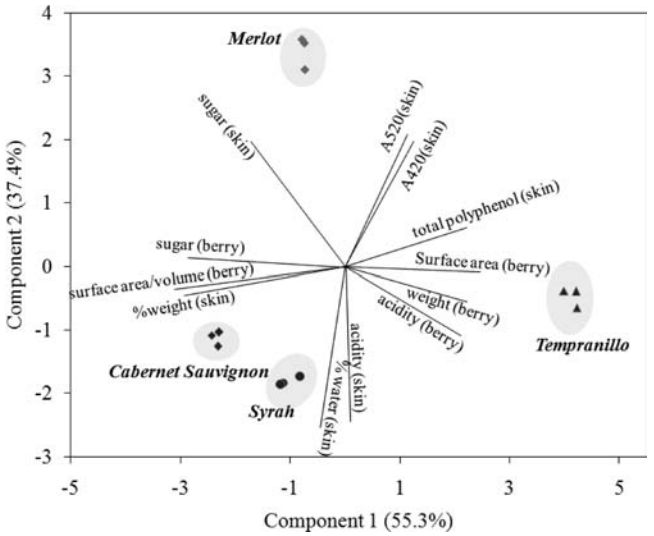

Figure 1. Principal component analysis. Biplot representation of variables and scores of the grapes studied.

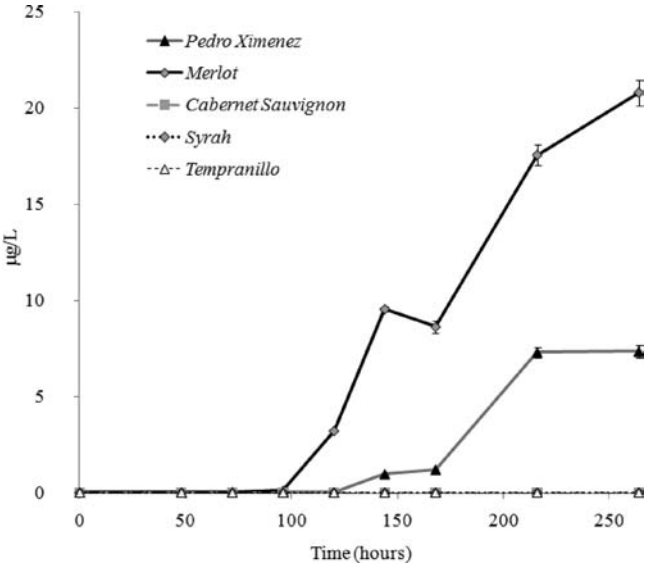

Figure 2. Ochratoxin A contents during growth of the *A. carbonarius* in red and white grapes.

skin that might somehow affect *A. carbonarius* early growth. These parameters were percent weight relative to berry weight, water and sugar contents, acidity, and total polyphenol index and brown and red phenol contents as determined from the absorbance at 420 and 520 nm, respectively. Although the main objective of this study was focused to obtain raisins from all grapes, the optimum harvest time for each variety had to be established on an individual basis. The criterion used was to harvest the grapes at their industrially optimum ripening time for standard winemaking rather than that for producing sweet wines. Therefore, the harvest date differed between the red varieties (Aug 11, 2009, for Merlot and Tempranillo, Aug 20 for Syrah, and Aug 24 for Cabernet Sauvignon) and also for the white variety (Aug 18, 2009).

As can be seen in **Table 1**, Pedro Ximenez grapes were those exhibiting the greatest mean berry weight (2.81 g) and highest surface area/volume ratio (3.99). Among red grapes, Tempranillo had the largest berries, followed by Merlot and Syrah (without significant difference between them), Cabernet Sauvignon providing the smallest berries with the lowest mean berry weight. The berries from the red varieties exhibited significantly different ($p < 0.001$) reducing sugar contents and titratable acidity, Syrah containing the greatest amounts of sugars (224 g/L) and Merlot the lowest acidity (40.3 mequiv/L against 76.4 for Cabernet Sauvignon, 90.4 mequiv/L for Syrah, and 160 mequiv/L for Tempranillo).

The grape varieties with the largest berries (Pedro Ximenez and Tempranillo) were also those exhibiting the lowest proportions of skin relative to total berry weight, as well as the lowest surface area/volume ratios. On the other hand, Merlot grape skin contained slightly less water (71.6% dry weight) than did the other three red varieties (74.7−75.7%) and the white variety Pedro Ximenez (80.5%). Likewise, Merlot grape skin was that having the highest sugar levels (186 mg/g), lowest acidity (0.051 mequiv/g), and highest proportions of brown and red compounds as measured in terms of A_{420} (0.168 au) and A_{520} (0.305 au). However, the highest total polyphenol content was that of Tempranillo (21.2 au at 280 nm).

In order to better observe the relationship among the measured parameters, the results were subjected to principal component analysis (PCA), accounting for 92.7% of the overall variance of the first two principal components (PCs) in combination. As can be seen from **Figure 1** the four red varieties placed in three different regions of the plane. The first grouped Tempranillo grapes, which exhibited the highest scores in PC1, grouping a second Merlot grapes, which had the highest scores in PC2, and a third for Cabernet Sauvignon and Syrah. PC1, which explained 55.3% of the overall variance, was influenced by the following factors in decreasing order: berry surface area/volume ratio (−0.387), berry surface area (0.385), total polyphenols in grape skin (0.366), berry weight (0.359), and percent skin weight (−0.347). Therefore, PC1 is related to berry morphology, it distinguishing mainly Tempranillo from the other red varieties. The greatest weight in PC2, which accounted for 37.4% of the variance, was that of acidity (−0.461), followed by the percent skin water (−0.454), A_{420} (0.404), A_{520} (0.419), and the skin content in reducing sugars (0.382). It suggests that PC2 is mainly related to chemical properties (particularly in grape skin) and discriminates Merlot grapes from the other red varieties.

On the other hand, the angles between the different eigenvectors of the PCA allowed the variables with the highest correlations to be identified, such correlations being confirmed by the corresponding Pearson *r* values and degrees of significance. Thus, statistically, the grape varieties containing the greatest contents of red phenols in their skin were also those having the highest concentrations of brown polymers ($r = 0.996$) and lowest water contents ($r = -0.856$). Likewise, the varieties with the highest skin sugar contents were also those exhibiting the lowest acidity ($r = -0.768$). Also, the varieties with the highest berry surface areas were those having the greatest berry weight ($r = 0.944$) and berry size (by effect of their low surface area/volume ratio; $r = -0.992$), lowest skin proportion ($r = -0.920$) and sugar content ($r = -0.729$), and highest acidity ($r = 0.824$).

Grape Inoculation and Ochratoxin A Production. Grapes of the Pedro Ximenez variety and the four red varieties were inoculated with a suspension of *A. carbonarius* and kept at 25 °C in order to monitor their ochratoxin A contents during growth of the fungus (the ochratoxin A was determined before inoculation, and no contents were found). The Cabernet Sauvignon, Syrah, and Tempranillo varieties exhibited no visible signs of fungal development. Therefore, ochratoxin A was only produced in Pedro Ximenez and Merlot grapes (**Figure 2**). As can be seen, these varieties that developed *A. carbonarius* exhibited no ochratoxin A production within the first 96 and 120 h, respectively, because this toxin is a secondary metabolite. Subsequently, during the

stationary phase of fungal growth the ochratoxin A concentration increased in an exponential way (to 20.81 μg/L in Merlot musts and 7.37 μg/L in Pedro Ximenez musts after 264 h of inoculation). The increased toxin production in the red variety relative to the white variety could be ascribed to the latter producing much larger berries, with a much lower proportion of skin and, in addition, with a much lower sugar content.

The higher resistance of some varieties to fungal attack can be ascribed to different factors. Thus, it may be a function of the ripening degree of the grapes. In this sense some authors (*23*) point out that ripe grapes are more liable to suffer fungi attack. However, the grapes studied in this work were all harvested in a similar ripening status coinciding with the industrial optimum for standard winemaking. Also, the sensitivity to the development of ochratoxin A producing fungi may be, at least partly, a characteristic of each grape variety as noted by authors such as Battilani et al. (*24*), although a later study by the same authors (*25*) shows that the harvest time is more influential than the particular grape variety. Other factors such as latitude, the climatic conditions of the year, presence of different fungi, crop techniques, and, particularly, fungicide use and application time may also have a considerable influence on the resistance of grapes to fungal growth (*13*). In this work the conditions for all of the grape varieties were the same, and as above-mentioned, the grapes were all treated with the same products and at the same times.

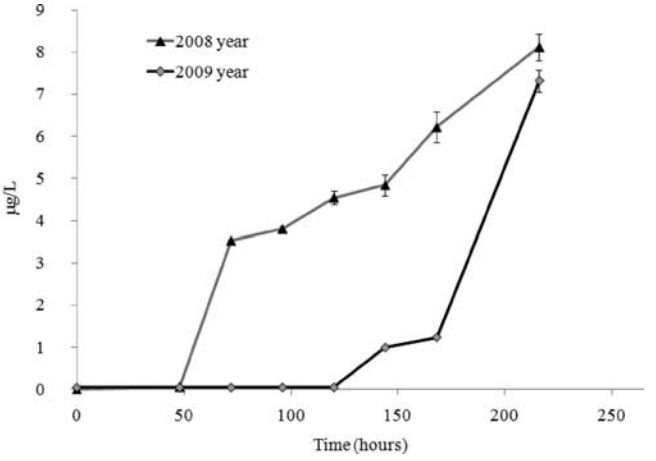

Figure 3. Ochratoxin A contents during growth of the *A. carbonarius* in two harvests (2008 and 2009) of Pedro Ximenez grapes.

In spite of the above comments, in order to better observe the potential influence on grape ripening because of different annual conditions (slight differences in weather and cropping techniques), Pedro Ximenez grapes were harvested from the same plot in two different consecutive years (2008 and 2009) and were inoculated with *A. carbonarius*. Although both grape batches developed the fungus, its growth was slower in the 2009 grapes, where ochratoxin A was detected only after 120 h versus 48 h in the 2008 grapes (**Figure 3**). However, the ochratoxin A levels found at the end of the experiment (216 h) were similar for both grapes (because of the faster increase for the 2009 year), which confirms that differences in climatic conditions and cropping techniques (mainly time of use of fungicides) can cause differences in the time for fungal development and, consequently, in ochratoxin A production rate. Accordingly, taking into account the above-mentioned similarities, one can reasonably ascribe differences in sensitivity to fungal growth between varieties to berry properties. In addition, because all grape varieties were harvested quite healthy, such sensitivity may be mainly dictated by skin properties. In this regard, Merlot grape skin exhibited the highest sugar content and lowest acidity, in addition to the lowest moisture content and highest absorbance at both 420 and 520 nm. However, none of these parameters can unequivocally account for the observed differences in sensitivity to fungal colonization. In addition, only acidity among all skin properties studied was found to be comparable between Merlot grapes and those of the white variety Pedro Ximenez (also fungus-sensitive), excluding the comparisons of phenol-related factors for obvious reasons.

Drying of Grapes Inoculated with *A. carbonarius*. The results obtained in the previous experiments allow to relate the fungus inoculation time with that required for its toxin to be detected. In order to examine changes in toxin production during the raisining process at a controlled temperature of 50 °C and relative humidity of 20%, as well as the effect of the dipping treatments, samples of Merlot and Pedro Ximenez grapes were inoculated with *A. carbonarius* prior to chamber-drying of grapes. The grapes were

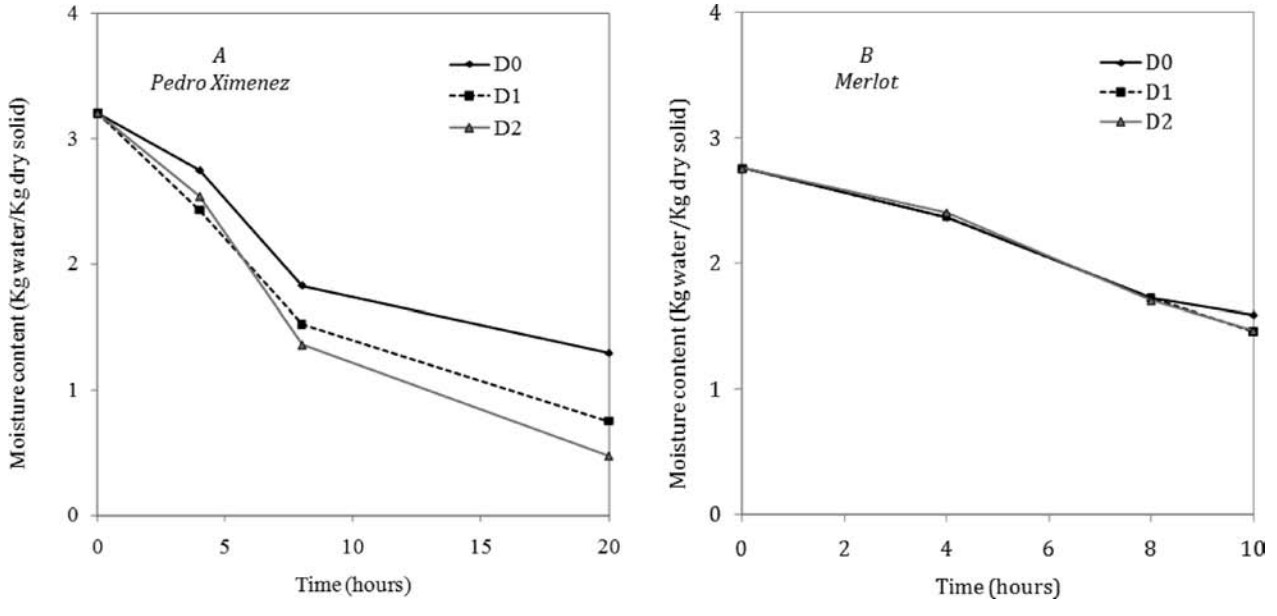

Figure 4. Drying curves of Pedro Ximenez (**A**) and Merlot (**B**) grapes.

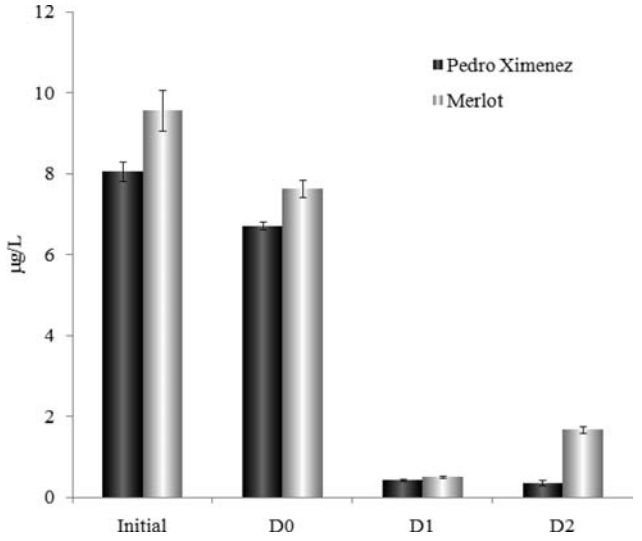

Figure 5. Contents in ochratoxin A of the initial musts and those obtained at the end of the grape-drying (Pedro Ximenez and Merlot varieties).

incubated at 25 °C, the time required to obtain an ochratoxin A concentration of 8−9 μg/L in their musts. The grapes of each variety were split into three batches that were subjected to no treatment (D0), dipping in olive oil + K_2CO_3 (D1), or dipping in an alkaline emulsion of ethyl oleate (D2). Dipping pretreatments have been applied for several years with a view to increasing grape skin permeability and facilitating moisture removal in order to expedite drying (*26*). The composition of the particular chemical agents used, their concentration, pH, and temperature, and the dipping time can be pointed out as the main factors governing the alteration of the skin microstructure and, consequently, their efficiency (*27*). **Figure 4** shows the drying curves obtained by plotting the grape moisture content (kilograms of water/kilograms of dry solid) versus drying time. All drying processes were stopped at the same time for each variety and weight losses measured in order to calculate the concentration factor resulting from water evaporation. Pedro Ximenez grapes lost after 20 h of drying 45.4%, 58.3%, and 64.9% of their weight for D0, D1, and D2, respectively. By contrast, Merlot grapes in 10 h lost 31.0% (D0), 34.4% (D1), and 34.3% (D2).

As can be seen from **Figure 5**, the initial concentrations of ochratoxin A in Pedro Ximenez and Merlot grapes (8.05 and 9.56 μg/L, respectively) decreased with drying; independently they were subjected or not to a dipping treatment. The grapes without dipping treatment exhibited a final ochratoxin A concentration of 6.72 μg/L (Pedro Ximenez) and 7.64 μg/L (Merlot), it showing a degradation of the toxin by the effect of heating at 50 °C in the chamber. Taking into account the concentration effect by loss of water during grape drying, this degradation was much more marked. In fact, the final ochratoxin A levels in the raisins should have been 1.8 (Pedro Ximenez) and 1.45 (Merlot) times higher than in the starting grapes if the changes had resulted exclusively from moisture losses. On the other hand, the grapes subjected to D1 exhibited much lower final ochratoxin A concentrations than the untreated grapes. Therefore, it is reasonable to attribute this decrease not only from degradation of the toxin but also from a partial mechanical removal of the fungus during dipping of the grapes prior to their drying in the chamber. Similar results were obtained for the dipping treatment D2, it confirming the mechanical removal effect. In spite of the better behavior of D1 and D2, both pretreatments must obviously have attacked the skin by effect of the contact with substances absent in its natural composition (potassium carbonate, olive oil, and ethyl oleate), which may have altered the sensory properties of the resulting raisins. These potential alterations were studied by a sensorial panel of experts in accordance with ISO 4121−1987 and ISO 5495−1983 norms, the taster being asked for color, aroma, and flavor of raisin musts. Based on the results, the musts from grapes treated with ethyl oleate were unanimously desirable with regard to color but undesirable in terms of aroma and flavor. On the other hand, the musts from untreated and olive oil-treated grapes were judged desirable in color, aroma, and flavor and very similar between them.

Overall, chamber-drying grapes under controlled temperature and humidity conditions with a view to obtaining raisins, showed one clear advantage over the traditional sun-drying process in terms of health. Thus, chamber-drying at 50 °C of untreated grapes stopped ochratoxin A production because of the inhibition in the growth of *A. carbonarius*, in addition to a partial degradation of the previously formed toxin as a result of heating. Certainly, the two dipping treatments resulted in more efficient removal of ochratoxin A and led to its virtually complete elimination. However, grape treatments increase raisining costs, and only the treatment with the olive oil emulsion was acceptable in relation to the sensory proprieties of the raisins. Therefore, this dipping treatment would only be indicated in grapes abnormally affected by toxin-producing fungi by effect of adverse climatic conditions of a particular year.

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