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Changes in Hydrophilic and Lipophilic Antioxidant Activity in Relation to their Phenolic Composition during the Chamber Drying of Red Grapes at a Controlled Temperature

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ABSTRACT: The purpose of this work was to study the variation of phenol compounds, as measured by HPLC, during the chamber drying under controlled temperature conditions of red grapes of the Merlot and Tempranillo varieties in relation to antioxidant activity. Both lipophilic and hydrophilic antioxidant activities in these grapes increased during the drying process; the former was measured via proton transfer in the coupled oxidation reaction between linoleic acid and β-carotene, and the second via electron transfer in the DPPH assay. The hydrophilic component was invariably greater in Tempranillo grapes, and so was the lipophilic component in Merlot grapes. Only the increase in hydrophilic antioxidant activity obtained a significant correlation with the phenolic compounds during the drying process. However, based on the phenolic fraction analysis, this result was primarily due to phenolic polymers and, to a lesser extent, also to phenolic acids, flavans, and some flavonols and anthocyans.

KEYWORDS: red grapes, grape drying, antioxidant activity, phenolic compounds

INTRODUCTION

Phenol compounds, which are among secondary plant metabolites, occur naturally in fruits and vegetables and are essential ingredients for the human diet. Grapes are highly appreciated for their high content in phenolic compounds, which contribute to the quality, color stability and sensory properties of table grapes and raisins as well as to those of wines made from them. The concentration of phenols in grapes depends on the particular variety, as well as on environmental factors and cultivation practices. As a rule, phenol compounds tend to accumulate in grape skins and seeds, but can also be found in grape pulp. Specifically, phenolic acids occur mainly in pulp, whereas flavonoid compounds usually concentrate in skins, seeds, and bunch stems.[1] The white grape skins abound with flavans, proanthocyanidins and hydroxycinnamic acids.[2] The red grape varieties also include anthocyanins and flavonols.[3] Interest in phenol compounds has grown substantially in recent years by virtue of their well-known healthy effects. Thus, the biological activity of these compounds has been examined in studies about their ability to inhibit the oxidation of low-density human lipoproteins,[4] their antioxidant properties and radioprotective effects,[5] and their ability to prevent cataracts,[6] neurodegenerative diseases, cardiovascular disturbances and cancer.[7]

Raisins are among the natural products containing the greatest amounts of phenol compounds, possessing the highest antioxidant activity. The phenol composition of raisins has been the subject of various studies[8,9] assessing their antioxidant properties and their relationship to phenols.[10,11]

The antioxidant activity in wine, grapes and raisins has been assessed with methods based on various reactions, both *in vitro* and *in vivo*. The *in vitro* assays used for this purpose are usually based on electron transfer reactions such as those exposing free-radical scavenging activity on ABTS[11,12] or on DPPH.[13−15] The *in vitro* assays also determine the reducing power (FRAP);[14,15] or the copper reduction (CUPRAC). Other alternative assays are based on proton transfer reactions and include the total peroxyl radical-trapping antioxidant (TRAP) assay, the oxygen radical absorbance capacity (ORAC) assay[13] and the coupled oxidation reaction between linoleic acid and β-carotene in a lipophilic medium.[16]

Lipophilic and hydrophilic assays provide complementary information.[16] The lipophilic assays are less effective with water-soluble compounds, although they are similar to the biological conditions. Hydrophilic assays are better to evaluate the antioxidant activity of compounds such as anthocyanins.[17] The linoleic acid/β-carotene assay is based on proton-transfer mechanisms and is especially relevant to the removal of free radicals *in vivo*. The DPPH assay measure the ability of antioxidants in wine to capture free radicals via electron-transfer mechanisms, which generates free radicals that are extraneous to the body.[16]

The drying process of grapes is traditionally used to obtain sweet wines. This process, used in southern Spain and Italy, is based on direct exposure of bunches to the sun or to the air. However, these processes are very dependent on weather conditions each year. In addition, these methods have risks due to attack by insects and fungi that produce phytotoxins. Therefore, in recent years various methods of drying chamber under controlled conditions have been developed, which show some interesting advantages against the traditional sun-drying of grapes. Principally, the drying time is shortened and chamber-drying allows selection of grapes at a higher ripening degree and more independent of the particular climatic conditions of

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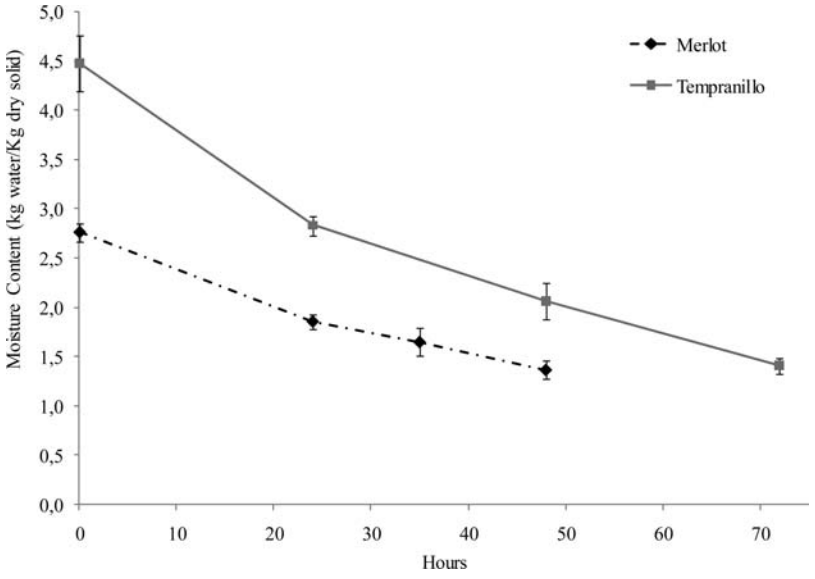

Figure 1. Drying curves of Merlot and Tempranillo grapes dried at 40 °C.

the year. Certainly, good quality raisins can be obtained from low ripeness grapes if dehydrated artificially, due to instantly stopping cellular respiration, in comparison with sun dried raisins.[18]

During grape dehydration, an increase in brown color of raisins takes place due to pigments formed by the effect of the enzymatic and nonenzymatic reactions.[8] Several phenolic compounds are well-known substrates for oxidative enzymes such as polyphenoloxidase (PPO) that catalyzes the oxidation of phenolic compounds to quinones in the presence of molecular oxygen, subsequently evolving to brown pigments (melanins). The Maillard reaction is faster with increasing temperatures (especially above 50 °C), and it is favored by pH values over the range 4−7, which are quite usual in foods.[19]

It is difficult to evaluate the contribution of each pathway to the browning of the grapes used in the production of sweet wines. On one hand, some authors[20] have pointed out that at the end of the ripening, and during drying, the grapes contain high concentrations of sugars that may inhibit the browning action of polyphenoloxidase, thereby gradually reducing the contribution of the enzymatic pathway as raisining progresses. On the other hand, the raisining temperature and the gradual decreasing of water activity of grapes can facilitate the progress of the Maillard reactions, leading to the formation of colored polymers of a high molecular weight.[21] Additionally, during drying at 40 °C the phenolic compounds (particularly anthocyans) gradually become more strongly colored polymeric pigments. Mazza and Maniati[22] found that the pH and the presence of oxygen, acetaldehyde, sulfur dioxide and copigment-forming molecules facilitate these reactions. In addition, these reactions may be favored by the drying temperature since other authors[23] previously found the fermentation temperature to be a critical trigger for the polymer pigment formation reactions.

In this work, we studied the variation of the phenolic composition of red grapes of the Merlot and Tempranillo varieties during their chamber drying at a controlled temperature in relation to their antioxidant activity. Hydrophilic antioxidant activity was measured via the DPPH assay and its lipophilic assay via the linoleic acid/β-carotene coupled oxidation reaction. The chamber-drying of red grape varieties under controlled conditions is a step in the elaboration of sweet red wines. This is intended to facilitate the diversification of the current supply of Andalusian red wines with a new one by introducing a nontraditional wine capable of reaching a market niche similar to that of Pedro Ximenez sweet white wine.

MATERIALS AND METHODS

Reagents. Anthocyanins (malvidin-3-*O*-glucoside chloride), phenolic acids (gallic, protocatechuic, *p*-OH-benzoic, vanillic, syringic, caffeic, *p*-coumaric, ferulic), catechins ((+)-catechin), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), DPPH (2,2,-diphenylpicrylhydrazyl), β-carotene, Tween 40 and linoleic acid were purchased from Sigma-Aldrich Chemical Co. (Madrid, Spain). Flavonols (quercetin, quercetin-3-glucoside, kaempferol, kaempferol-3-rutinoside, kaempferol-3-glucoside, myricetin, isorhamnetin, isorhamnetin-3-glucoside) were obtained from Extrasynthese (Genay, France). Methanol, ethanol, formic acid, chloroform, hydrochloric acid, ethyl acetate, acetonitrile and purified water were purchase from Merck (Madrid, Spain).

Grape Drying. The material used in this study consisted of Tempranillo and Merlot grapes from the Montilla-Moriles region (southern Spain). For each variety was selected an area of 5 × 5 vines for harvesting grapes in 2009. An amount of about 15 kg of grapes was uniformly distributed in several trays and allowed to dry in a Frisol Climatronic chamber at an air temperature of 40 °C and a constant relative humidity of *ca.* 20%. All drying tests were done in triplicate. During the drying process, appropriate samples of the two grape varieties were withdrawn for the measurement of their weight loss and content in reducing sugars. Drying was stopped when the sugar content fell below 325 g/L approximately. Once in the laboratory, the whole bunches of raisins were pressed on a vertical press similar to industrial models. The maximum pressure reached in each pressing cycle was 300 bar, and each raisin batch was pressed in two cycles. Without skin maceration, the percentage of must obtained was 35 and 25% (volume/weight) for Tempranillo and Merlot respectively, which was centrifuged at 3000 rpm for analysis.

Extraction of Phenolic Compounds. A volume of 2 mL of must was passed through a Sep-Pak C18 cartridge, with 900 mg of filling

(Long Body Sep-Pak Plus; Waters Associates; Milford, MA) that was previously activated with 10 mL of methanol and 10 mL of HCl 0.01% in water. The cartridge was eluted with 10 mL of HCl 0.01% in water. This volume in addition to the volume obtained as a result of the sample run-through prior to the elution was used for the determination of phenolic acids, esters and flavan-3-ol fraction (fraction 1). The flavonol fraction was eluted with 5 mL of ethyl acetate (fraction 2). The anthocyanin fraction and polymers was eluted with 5 mL of methanol (fraction 3). These three collected fractions were concentrated and passed through a filter of 0.45 μm pore size for injection into a Spectra-Physics (San Jose, CA) P4000 HPLC instrument. All the fractions were concentrated on a rotary evaporator to 2 mL.

Identification and HPLC Analysis. The identification of the phenolic compounds was achieved by comparison of the retention times of the standards, UV spectra obtained by diode array HPLC (Spectra-Physics UV6000LP) and calculation of UV absorbance ratios after coinjection of samples and standards one at a time. Peak-height comparison was based on the results of samples with and without the standard. The identification of compounds was confirmed by HPLC/ESI-MS analysis (TermoQuest Finnigan AQA quadrupole mass spectrometer). The instrument was operated in both the negative ion and positive ion modes. The ion spray voltage was −4 kV and the orifice voltage −60 V. Mass data were acquired in two different ways, namely, in the scan mode (by scanning the m/z range 150−1066 at 1.2 intervals) and in the multiple ion mode (by using mass ranges around specific m/z values). Caftaric and coutaric acids were isolated by the method described by Singleton et al.[24] The purity of standards was 95−99%. Each compound was quantified by comparison with a calibration curve obtained with the corresponding standard, except the caftaric, coutaric and feftaric acid, which were quantified as caffeic, *p*-coumaric and ferulic acid, respectively, and procyanidins, which were quantified as catechin. Anthocyanins were quantified as malvidin-3-glucoside. The fraction of polymers has been mostly identified as anthocyanin compounds of high molecular weight by its absorption at 520 nm, but it may include other compounds belonging to other phenolic families.

The column used in the analyses was a 250 mm × 4.6 mm i.d., 5 μm, LiChrospher 100 RP-18, using 10% aqueous formic acid (A) and acetonitrilo/formic acid/H_2O (45:45:10) (B) as mobile phases at a flow rate of 1 mL/min.

The elution phases and detection were as follows.

Fraction 1: gradient elution from 5 to 10% B in 35 min, gradient elution up to 20% B in 10 min, gradient elution up to 30% B in 10 min, gradient elution up to 100% CH_3CN in 10 min, and isocratic elution for 10 min; detection at 280 nm for quantification of phenolic acids and flavan-3-ol and 315 nm for quantification of esters of hydroxycinnamic acid;

Fraction 2: gradient elution from 5 to 30% B in 5 min, gradient elution up to 40% B in 14 min, gradient elution up to 80% B in 11 min, gradient elution up to 100% CH_3CN in 1 min, and isocratic elution for 10 min; detection at 360 nm for quantification of flavonols;

Fraction 3: gradient elution from 15 to 30% B in 17 min, gradient elution up to 73% B in 28 min, gradient elution up to 100% B in 3 min, and isocratic elution for 10 min;

Polymer fraction: gradient elution from 5 to 10% B in 5 min, gradient elution up to 20% B in 10 min, gradient elution up to 30% B in 10 min, gradient elution up to 100% B in 15 min, and isocratic elution for 10 min; detection at 520 nm for quantification of anthocyanins and 280 nm for quantification of polymers fractions.

Determination of Antioxidant Activity. Antioxidant activity was analyzed in the musts obtained during the drying of the Merlot and Tempranillo grapes and in the previous cited fraction through two methods:

DPPH Assay. The ability of the musts to scavenge free radicals was determined according to Alen-Ruiz et al.[16] For this purpose, a 45 mg/L solution of DPPH (2,2-diphenylpicrylhydrazyl) in methanol was prepared on a daily basis and stored in the dark. All musts were diluted 10 times with a solution containing 12% ethanol in water prior to analysis. A 80 mg/L solution of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (a vitamin E analogue) was used as a standard. The analytical procedure was as follows: a 200 μL aliquot of diluted must was placed in a cell to which 3 mL of a 45 mg/L solution of DPPH in methanol was then added. A blank (200 μL dilution sample + 3 mL methanol), a control sample (200 μL of 12% ethanol in water + 3 mL of DPPH solution) and a Trolox standard (200 μL of Trolox solution + 3 mL of DPPH solution) were also prepared in parallel. Following vigorous stirring, the absorbances at 517 nm of the control sample and blank were measured on a Perkin-Elmer Lambda 25 spectrophotometer. The sample and the Trolox standard were measured under identical conditions after 10 min of incubation at room temperature. All samples were analyzed in triplicate. The results, expressed in millimoles of Trolox (mmol TE) per liter, were calculated as follows:

$$\text{antioxidant activity of sample (mmol TE/L)} = (0.32A_1 \times \text{wine dilution factor})/A_2$$

$$A_1 = \text{absorbance}_{\text{control}(t=0)} - \text{absorbance}_{\text{sample}}$$

$$A_2 = \text{absorbance}_{\text{control}(t=0)} - \text{absorbance}_{\text{standard}(t=0)}$$

$$A_{\text{sample}} = \text{absorbance}_{\text{sample}(t=10)} - \text{absorbance}_{\text{blank}(t=0)}$$

Linoleic Acid/β-Carotene Coupled Oxidation Reaction. Antioxidant activity in the musts was assessed with the β-carotene linoleate model system.[17] For this purpose, a solution of β-carotene was prepared by dissolving 2 mg of the compound in 10 mL of chloroform. A 2.5 mL aliquot of the solution was placed in a 100 mL round-bottom flask and supplied with 60 mg of linoleic acid and 400 mg of Tween 40 as emulsifier. Following the vacuum removal of the chloroform, the flask was filled with 100 mL of aerated distilled water and stirred vigorously. Then, several cells were filled with 3 mL of emulsion containing a 200 μL aliquot of must diluted to a variable extent with 12% of ethanol in distilled water. A control sample was also prepared in parallel. Absorbance measurements (470 nm) were made at t = 0 and after incubation at 50 °C for 60 min. Antioxidant activity was expressed as the percent of inhibition with respect to the control sample and calculated as follows:

$$\%\text{inhibition} = (\Delta A_{\text{control}} - \Delta A_{\text{sample}})/\Delta A_{\text{control}} \times 100$$

where

$$\Delta A_{\text{control}} = A_{\text{control}(t=0)} - A_{\text{control}(t=60)}$$

$$\Delta A_{\text{sample}} = A_{\text{sample}(t=0)} - A_{\text{sample}(t=60)}$$

Antioxidant activity was estimated from the slopes of the linear portions of the regression curves obtained by plotting the percent of inhibition against the must volume. The highest slope corresponded the highest antioxidant activity.

Statistical Procedures. The results for all samples were subjected to triplicate simple regression analysis and variance analyses (ANOVA) by using the Statgraphics Computer Package v. 5.0 from Statistical Graphics Corp.

Table 1. Concentrations (mg/L) of Phenolic Compounds (Means and Standard Deviations) of Musts Obtained During Grape Drying and Homogeneous Groups

	Merlot			Tempranillo			
	0 h	24 h	48 h	0 h	24 h	48 h	72 h
Phenolic Acids							
gallic acid	1.27 ± 0.026 a	2.03 ± 0.180 b	3.82 ± 0.081 c	1.06 ± 0.006 a	1.98 ± 0.015 b	4.88 ± 0.630 c	13.53 ± 0.115 d
protocatechuic acid	1.91 ± 0.101 a	2.15 ± 0.428 a	2.02 ± 0.251 a	1.32 ± 0.099 b	0.546 ± 0.103 a	2.67 ± 0.604 c	3.36 ± 0.175 d
p-OH-benzoic acid	0.630 ± 0.077 a	1.09 ± 0.161 b	1.25 ± 0.090 b	nd	nd	nd	nd
vanillic acid	nd	2.05 ± 0.050	3.03 ± 0.224	nd	nd	0.91 ± 0.114 a	0.87 ± 0.065 a
syringic acid	1.04 ± 0.118 a	0.940 ± 0.107 a	1.37 ± 0.129 b	nd	nd	0.44 ± 0.040 a	0.37 ± 0.033 a
o-coumaric acid	0.070 ± 0.010 a	0.100 ± 0.009 a	0.070 ± 0.011 a	nd	nd	0.22 ± 0.022 a	0.38 ± 0.050 b
p-coumaric acid	0.540 ± 0.013 a	0.580 ± 0.022 a	0.590 ± 0.019 a	nd	0.56 ± 0.014	0.58 ± 0.018 a	0.58 ± 0.010 a
total	5.46 ± 0.111 a	8.95 ± 0.367 b	12.1 ± 0.591 c	2.44 ± 0.158 a	3.08 ± 0.132 b	9.70 ± 0.233 c	19.1 ± 0.350 d
Esters							
c-caftaric acid	0.630 ± 0.079 a	0.810 ± 0.046 b	0.850 ± 0.040 b	0.620 ± 0.013 a	0.62 ± 0.004 a	0.71 ± 0.054 b	0.96 ± 0.025 c
t-caftaric acid	1.18 ± 0.036 b	1.04 ± 0.101 ab	0.910 ± 0.141 a	1.57 ± 0.107 c	0.85 ± 0.005 a	1.60 ± 0.050 c	1.14 ± 0.010 b
c-coutaric acid	0.580 ± 0.024 a	0.620 ± 0.013 b	0.630 ± 0.025 b	1.23 ± 0.015 c	1.08 ± 0.010 b	1.03 ± 0.064 b	0.87 ± 0.035 a
t-coutaric acid	0.530 ± 0.004 a	0.630 ± 0.028 b	0.810 ± 0.065 c	0.880 ± 0.012 c	0.86 ± 0.004 c	0.71 ± 0.017 a	0.77 ± 0.013 b
c-feftaric acid	0.490 ± 0.019 c	0.310 ± 0.009 b	0.220 ± 0.040 a	1.27 ± 0.067 b	1.00 ± 0.014 a	1.23 ± 0.025 b	0.99 ± 0.082 a
t -feftaric acid	0.090 ± 0.022 a	0.110 ± 0.017 b	0.130 ± 0.041 c	0.060 ± 0.016 a	0.17 ± 0.025 b	0.21 ± 0.013 c	0.25 ± 0.016 d
total	3.49 ± 0.081 a	3.51 ± 0.023 a	3.56 ± 0.176 a	5.64 ± 0.197 c	4.57 ± 0.026 a	5.49 ± 0.101 c	4.98 ± 0.062 b
Flavans							
(+)-catechin	5.71 ± 0.067 a	11.3 ± 0.096 b	10.5 ± 0.092 b	3.99 ± 0.537 a	10.69 ± 1.13 b	15.10 ± 1.42 c	13.26 ± 0.635 c
(−)-epicatechin	14.3 ± 0.306 a	38.2 ± 1.25 c	57.2 ± 0.577 c	44.9 ± 1.50 a	43.63 ± 0.423 a	81.47 ± 0.451 b	75.23 ± 2.95 c
procyanidin B1	3.00 ± 0.096 a	10.7 ± 1.31 b	5.38 ± 0.465 b	21.1 ± 0.378 a	35.15 ± 0.071 b	37.40 ± 1.56 b	19.97 ± 0.635 a
procyanidin B2 + B4	4.69 ± 0.121 b	6.55 ± 0.102 a	4.19 ± 0.357 a	7.84 ± 0.555 a	9.76 ± 0.784 b	11.47 ± 0.252 c	11.83 ± 1.07 c
epigalocatequin gallate	0.440 ± 0.051 c	0.140 ± 0.050 b	0.330 ± 0.004 b	0.270 ± 0.052 a	0.55 ± 0.041 b	0.398 ± 0.050 ab	0.391 ± 0.015 ab
epicatechin gallate	0.640 ± 0.034 a	1.78 ± 0.311 a	0.850 ± 0.060 a	0.150 ± 0.005 a	0.64 ± 0.037 a	1.91 ± 0.085 b	2.93 ± 0.042 b
total	28.8 ± 0.926 a	68.8 ± 2.21 b	78.4 ± 1.86 c	77.7 ± 3.01 a	96.3 ± 8.24 b	144 ± 4.97 d	123 ± 1.62 c
Flavonols							
quercetin-3-glucoside	1.82 ± 0.012 a	2.43 ± 0.021 b	4.32 ± 0.121 c	0.55 ± 0.005 a	2.49 ± 0.040 b	4.32 ± 0.129 d	3.05 ± 0.036 c
kaempferol-3-rutinoside	0.15 ± 0.016 a	0.36 ± 0.006 c	0.33 ± 0.013 b	0.22 ± 0.003 a	0.67 ± 0.082 c	0.99 ± 0.016 d	0.38 ± 0.009 b
kaempferol-3-glucoside	0.30 ± 0.015 a	0.67 ± 0.004 b	0.82 ± 0.014 c	0.43 ± 0.003 a	3.06 ± 0.235 c	2.15 ± 0.062 b	1.98 ± 0.071 b
myricetin	0.02 ± 0.002 a	0.10 ± 0.002 b	0.16 ± 0.007 c	0.01 ± 0.002 a	0.07 ± 0.011 c	0.14 ± 0.007 d	0.04 ± 0.006 b
isorhamnetin-3-glucoside	0.58 ± 0.009 a	2.37 ± 0.012 b	4.95 ± 0.197 c	0.16 ± 0.004 a	1.53 ± 0.047 c	1.35 ± 0.051 b	1.34 ± 0.006 b
quercetin	nd	nd	0.78 ± 0.135 a	nd	0.23 ± 0.013 a	0.39 ± 0.015 b	0.65 ± 0.025 c
kaempferol	nd	0.14 ± 0.004 a	0.29 ± 0.013 b	nd	0.17 ± 0.003 a	0.24 ± 0.004 b	0.38 ± 0.011 c
isorhamnetin	nd	nd	0.22 ± 0.021 a	nd	nd	0.02 ± 0.003 a	0.06 ± 0.002 b
total	3.83 ± 0.032 a	8.67 ± 0.055 b	14.2 ± 0.481 c	1.72 ± 0.014 a	10.7 ± 0.252 b	14.2 ± 0.403 c	10.5 ± 0.182 b
Anthocyanin-3-glucosides							
delphinidin-3-glucoside	3.11 ± 0.091 a	7.49 ± 0.272 c	5.92 ± 0.181 b	2.15 ± 0.006 a	16.3 ± 0.651 d	11.3 ± 0.404 c	7.58 ± 0.123 b
cyanidin-3-glucoside	4.06 ± 0.150 a	6.97 ± 0.182 c	6.11 ± 0.150 b	3.17 ± 0.057 a	7.28 ± 0.169 d	6.68 ± 0.229 c	5.34 ± 0.074 b
petunidin-3-glucoside	3.86 ± 0.802 a	12.4 ± 1.803 c	9.64 ± 2.27 b	2.52 ± 0.010 a	25.2 ± 0.709 d	19.0 ± 0.862 c	11.4 ± 0.100 b
peonidin-3-glucoside	12.9 ± 0.395 a	44.1 ± 0.874 c	31.20 ± 1.14 b	5.22 ± 0.113 a	16.5 ± 0.115 c	17.4 ± 1.34 c	11.1 ± 0.153 b
malvidin-3-glucoside	23.5 ± 1.106 a	116 ± 4.271 c	99.7 ± 5.40 b	19.5 ± 0.551 a	149 ± 5.682 d	127 ± 8.303 c	77.5 ± 0.551 b
total	47.2 ± 2.26 a	188 ± 6.71 c	153 ± 8.38 b	32.6 ± 0.708 a	214 ± 7.17 d	182 ± 11.1 c	113 ± 0.797 b
Anthocyanin-3-acetylglucosides							
delphinidin-3-acetylglucoside	2.23 ± 0.006 a	4.96 ± 0.457 b	4.42 ± 0.112 b	2.07 ± 0.017 a	4.98 ± 0.229 b	5.37 ± 0.209 c	5.02 ± 0.006 b
cyanidin-3-acetylglucoside	2.10 ± 0.012 a	4.64 ± 0.010 b	4.55 ± 0.100 b	2.06 ± 0.006 a	4.22 ± 0.029 bc	4.25 ± 0.089 c	4.15 ± 0.031 b
petunidin-3-acetylglucoside	2.49 ± 0.020 a	5.99 ± 0.099 c	5.34 ± 0.115 b	2.09 ± 0.012 a	5.49 ± 0.036 d	5.02 ± 0.021 c	4.54 ± 0.049 b
peonidin-3-acetylglucoside	4.25 ± 0.130 a	11.0 ± 0.200 c	8.72 ± 0.376 b	2.18 ± 0.000 a	7.16 ± 0.160 d	6.46 ± 0.155 c	5.23 ± 0.173 b
malvidin-3-acetylglucoside	7.86 ± 0.280 a	34 ± 0.379 c	26.9 ± 0.929 b	3.06 ± 0.023 a	13.5 ± 0.416 d	11.5 ± 0.404 c	8.61 ± 0.202 b
total	18.9 ± 0.445 a	60.6 ± 1.08 c	50.0 ± 1.53 b	11.5 ± 0.041 a	35.3 ± 0.797 d	32.6 ± 0.445 c	27.5 ± 0.382 b

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Table 1. Continued

	Merlot			Tempranillo			
	0 h	24 h	48 h	0 h	24 h	48 h	72 h
Anthocyanin-3-coumaroylglucosides							
cyanidin-3-coumaroylglucoside	2.10 ± 0.017 a	4.56 ± 0.040 c	4.42 ± 0.025 b	2.08 ± 0.006 a	4.61 ± 0.172 b	4.52 ± 0.173 b	4.37 ± 0.090 b
petunidin-3-coumaroylglucoside	2.12 ± 0.026 a	4.53 ± 0.051 b	4.47 ± 0.035 b	2.10 ± 0.015 a	7.45 ± 0.051 d	6.39 ± 0.136 c	5.28 ± 0.017 b
peonidin-3-coumaroylglucoside	2.35 ± 0.015 a	4.80 ± 0.159 b	4.78 ± 0.045 b	2.15 ± 0.015 a	5.15 ± 0.040 d	5.01 ± 0.096 c	4.55 ± 0.044 b
malvidin-3-coumaroylglucoside	2.67 ± 0.038 a	6.97 ± 0.163 c	6.68 ± 0.051 b	2.68 ± 0.038 a	18.0 ± 0.289 d	14.8 ± 0.737 c	10.3 ± 0.000 b
total	9.24 ± 0.085 a	20.9 ± 0.236 c	20.4 ± 0.068 b	9.02 ± 0.035 a	35.2 ± 0.162 d	30.7 ± 1.14 c	24.5 ± 0.073 b
Anthocyanin-3-caffeoylglucosides							
malvidin-3-caffeoylglucoside	2.17 ± 0.000 a	6.13 ± 0.042 b	6.14 ± 0.051 b	2.21 ± 0.006 a	7.67 ± 0.116 c	8.35 ± 0.251 d	6.93 ± 0.044 b
total	2.17 ± 0.000 a	6.13 ± 0.042 b	6.14 ± 0.051 b	2.21 ± 0.006 a	7.67 ± 0.116 c	8.35 ± 0.251 d	6.93 ± 0.044 b
Polymers							
polymers	5.46 ± 0.763 a	162 ± 5.76 b	255 ± 5.35 c	89.2 ± 3.60 a	298 ± 11.5 b	280 ± 10.0 b	432 ± 26.1 c
total phenolic compounds	125 ± 1.70 a	527 ± 9.27 b	592 ± 5.21 c	232 ± 7.47 a	705 ± 15.3 b	707 ± 7.19 b	761 ± 28.5 c

RESULTS AND DISCUSSION

Grape Drying. Figure 1 shows the drying curves for the two grape varieties as obtained by plotting their moisture contents as a function of time in hours. As can be seen, the initial moisture content differed between the two varieties. Thus, it was 2.76 kg water/kg dry matter for Merlot grapes and 4.47 kg water/kg dry matter for Tempranillo grapes. The respective sugar contents were 205.7 and 193.1 g/L. As a result, the grape drying time needed to obtain the target sugar concentration in the musts differed between Merlot and Tempranillo (48 h for a content of 334.2 g/L in the former and 72 h for 323.2 g/L in the latter).

The moisture content of the grapes decreased gradually during drying, but more markedly in the Tempranillo grapes since the process was stopped at a very similar moisture level (1.37 kg water/kg dry solid for Merlot grapes and 1.41 kg water/kg dry solid for Tempranillo grapes). A linear fit of the data provided a drying rate of −0.029 (R^2 = 97.5%) for Merlot and −0.043 (R^2 = 94.8%) for Tempranillo. The resulting increase in sugar content was similar for both types of grapes (1.62 times for Merlot and 1.67 times for Tempranillo). These values were used as references for the losses through water evaporation in all compounds, which were assumed to undergo no reaction.

Phenolic Compounds. Table 1 shows the variation of the contents in phenol compounds (means and standard deviations), expressed in milligrams-per-liter, of musts obtained from grapes dried in the chamber. As can be seen, the initial phenol profile differed between the two grape varieties. Thus, the Merlot must exhibited higher contents in phenolic acids, flavonols, glycosylated anthocyans and catechin, whereas the Tempranillo must had greater concentrations of hydroxycinnamic esters and flavan-3-ols with the exception of (+)-catechin. It is worthy to note the fact that the polymer fraction was much more important in Tempranillo (89.2 mg/L) than in Merlot (5.46 mg/L). On the other hand, the concentrations of cumaroylglucosides and caffeoylglycosides of anthocyans were similar for both varieties. Overall, anthocyans were the major compounds for both types of grapes, and flavonols and phenolic acids were present in lower proportions than flavans, which is consistent with previous results for other red grape varieties.[25]

Since the drying process caused the grapes to lose substantial amounts of water, all grape components should have gradually increased in concentration. However, the net outcome for some phenolic contents is a balance between concentration gains and losses. Some phenols can take part in different types of reactions including nonenzymatic browning and/or autoxidation, and enzymatic oxidation reactions involving polyphenol oxidases or peroxidases, all of which reduce their concentrations. In addition, it is known that some flavan-3-ol high molecular weight derivatives can be hydrolyzed to phenolic compounds of lower molecular weights, increasing the contents in the latter.[18] Finally, in addition the musts were enriched with phenolic compounds from the grape skins, because the drying process altered the grape skins and facilitated the extraction of phenols by the effect of strong pressing of the raisins to obtain the musts.

The total concentrations of phenolic acids increased to a greater extent (2.21 times for Merlot and 7.82 times for Tempranillo) than did those of reducing sugars (1.62 times for Merlot and 1.67 times for Tempranillo), used as references for moisture losses, in both grape varieties. The increase was mainly due to gallic acid, the concentration of which rose 3.0 and 12.7 times in Merlot and Tempranillo, respectively.

All hydroxycinnamic esters except that of *t*-coutaric acid exhibited a decrease or no change in their concentrations in the drying process of the Merlot grapes. Such concentrations, however, never reached the levels expected due to water losses. Therefore, the esters, which are highly suitable substrates for polyphenol oxidases, must have undergone degradation reactions. Tempranillo grapes behaved similarly in this respect, where all esters remained unchanged, except for *c*-caftaric and *t*-feftaric acids, which exhibited a slight increase in concentration. In fact, the first step in the enzymatic oxidation process is the oxidation of *t*-caftaric acid to the corresponding quinone in the presence of polyphenol oxidase (PPO) as a catalyst. This acid, however, can be regenerated by the oxidation of reductants such as ascorbic acid or sulfite present in the medium.[26]

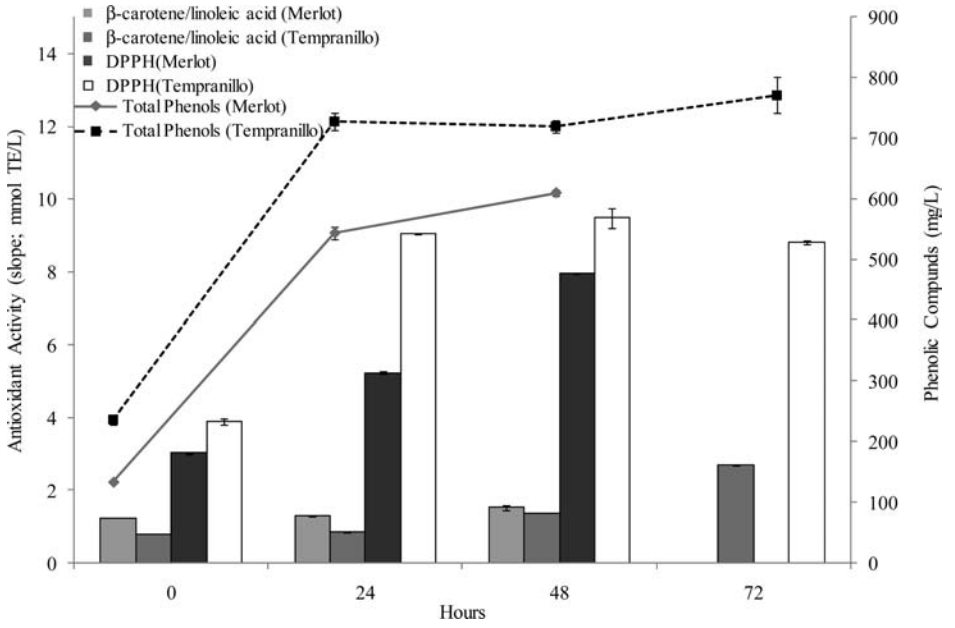

Figure 2. Changes in the total phenolic compounds contents and their antioxidant activity measured by DPPH and linoleic acid/β-carotene assay during the drying of the two grape varieties.

The amounts of flavan-3-ol compounds increased 2.73 times during the drying process in Merlot grapes. However, all the compounds in this fraction except for (−)-epicatechin exhibited a decrease in concentration after 24 h. Drying also increased the contents in these compounds of the Tempranillo must, by a factor of 1.66. Nevertheless, this increase lasted only 48 h, after which the concentrations in flavan-3-ol derivatives started to decrease. In addition to the above-described concentration effect of water evaporation from the grapes, these results may be explained by the hydrolysis of some high molecular weight flavan-3-ol derivatives producing smaller compounds.[27] The effect may also have been the result of additional reactions reducing the concentrations of these compounds, which are known to be good substrates for polyphenol oxidases or peroxidases,[28] although less reactive than hydroxycinnamic acids, which would have been the first to be degraded. In any case, flavans can react with *o*-quinones previously formed from hydroxycinnamic acids.[29] In addition, these compounds can polymerize together with some anthocyanins to give colored pigments and take part in other processes such as nonenzymatic browning and/or autoxidation reactions. Thus, Karadeniz et al.[8] found flavan-3-ol derivatives to be completely degraded during the raisining of grapes at increased temperatures. In this study the grapes were not dehydrated completely and the degradation was incomplete. In fact, the net effect was an increase in concentration in these compounds.

Flavonols increased in concentration during the drying of Merlot grapes; the increase, however, exceeded the level expected due to the water evaporation, which suggests that some compounds may have been extracted from the grape skins during drying and subsequent pressing of the grapes. In fact, flavonols occur naturally in this grape fraction. Tempranillo must have also exhibited an increase in flavonols throughout the grape drying process; most of the compounds, however, exhibited a decrease at the end of the process, which suggests that they must have undergone some degradation in parallel to their extraction.

All compounds in the anthocyan fraction exhibited an increase in concentration at an early stage of drying in both grape varieties and then a decrease at the end of the process; in any case, the final concentrations exceeded the starting levels in both types of grapes. The increase was much greater than expected because of water evaporation alone, so it must be additionally ascribed to the extraction of anthocyans from the grape skins during the drying process. It was observed that the pulp color in both varieties increased with drying, and even more so after pressing. Following marked extraction at the beginning in both types of grapes, the anthocyan contents exhibited a decrease possibly due to copigmentation reactions between themselves (self-association) or with other molecules including alkaloids, aminoacids, nucleotides, carbohydrates and phenol compounds (intermolecular copigmentation).[30] In addition, these compounds can take part in various reactions such as the copolymerization of anthocyanins with quinone−phenol condensation products, which prevent their oxidation.[31] Anthocyans, flavans and flavonols are poor substrates for PPO. However they can be rapidly altered by enzymatically produced quinones via coupled oxidation reactions or condensation between phenols and quinones.[29]

Phenolic polymers exhibited a marked increase during drying of both types of grapes, but much more markedly in Merlot must (from 5.46 to 254.77 mg/L, i.e. 46.7 times) than in Tempranillo must (from 89.2 to 431.9 mg/L, 4.82 times). These results suggest that phenol compounds (particularly anthocyans) gradually become polymeric pigments during drying at 40 °C.

Antioxidant Activity. Figure 2 shows the variation of antioxidant activity as measured with the DPPH assay and the coupled oxidation reaction between linoleic acid and β-carotene (slope of the linear portion of the curve) during drying of the two grape varieties. The figure also shows the variation of the total contents in phenolic compounds as the combined concentration of all phenols identified. These assays measure the ability of antioxidants present in the must to scavenge free radicals via an electron-transfer (DPPH assay) or proton-transfer mechanism (linoleic acid/β-carotene reaction), i.e. the former measures hydrophilic antioxidants and the latter lipophilic antioxidants.[32] The DPPH values of antioxidant activity in must from the initial

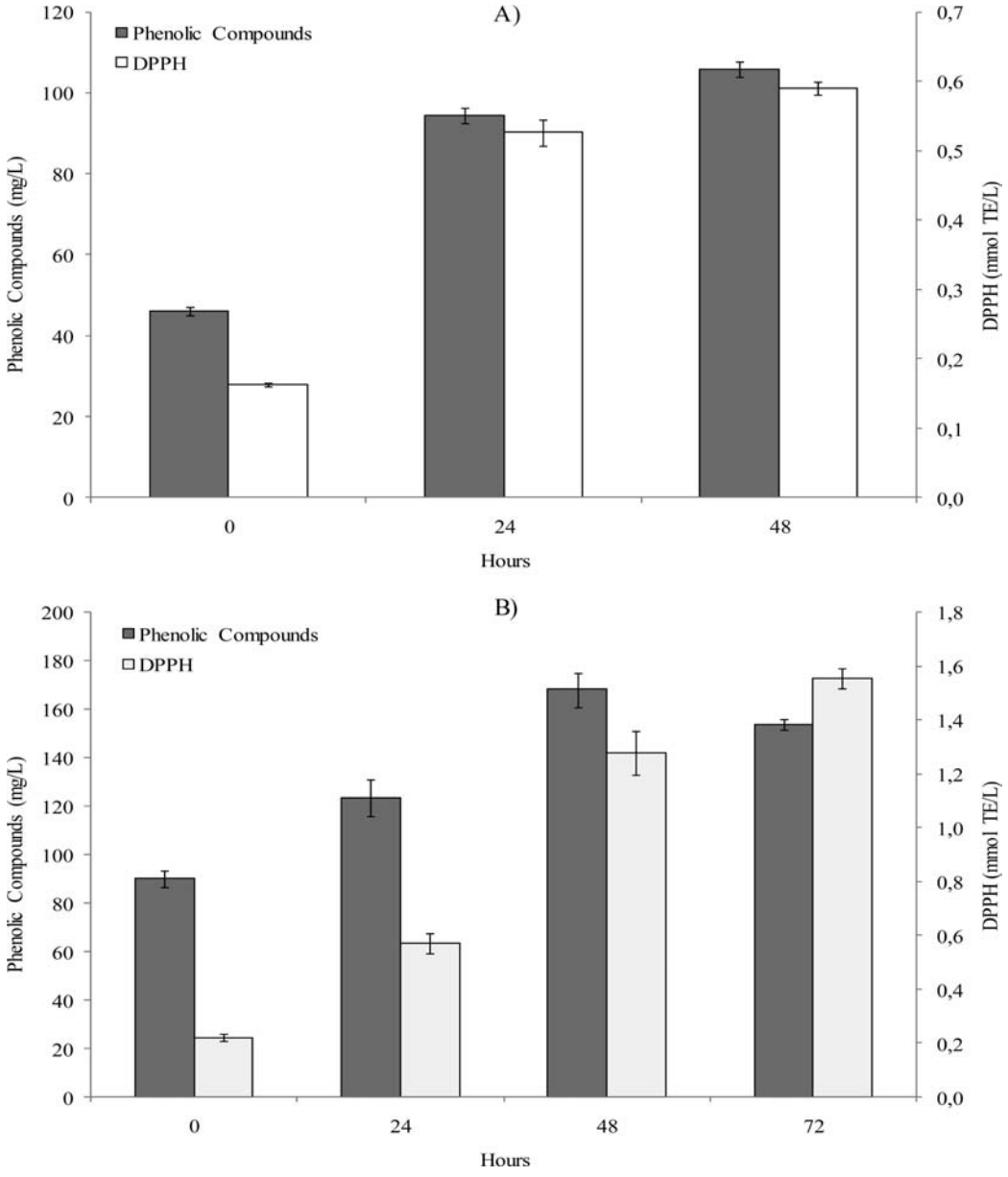

Figure 3. Antioxidant activity measured with the DPPH assays and combined concentration of the phenol compounds of fraction 1 from Merlot (A) and Tempranillo (B) grapes.

grapes were 3.02 mmol TE/L for Merlot and 3.90 mmol TE/L for Tempranillo, which were similar to others previously reported[16] for young and slightly aged red wines (3 and 5 mmol TE/L, respectively). The final values for the must from raisins (7.94 mmol TE/L for Merlot and 8.80 mmol TE/L for Tempranillo) were closer to those for older wines, which exhibit an antioxidant activity of 7−8 mmol TE/L.[13] Also, the must from the initial grapes exhibited a higher lipophilic antioxidant activity in the Merlot variety (1.21) than in the Tempranillo variety (0.80), the latter being that containing the higher concentrations of phenols.

Antioxidant activity as measured with the two assays increased throughout the drying process in both grape varieties. Hydrophilic antioxidant activity was invariably higher in the Tempranillo variety and lipophilic activity in the Merlot variety. The increase should have resulted from that in phenolic compounds during the drying process (from 125 to 592 mg/L in Merlot and 232 to 761 mg/L in Tempranillo as measured by HPLC). The total polyphenol value has been widely used to correlate antioxidant activity in red musts and wines.[1,4,14]

A linear regression analysis of the total phenol contents and antioxidant activity values provided by the two assays revealed a significant correlation between the former and the DPPH values (R^2 = 0.8961, p < 0.001). This suggests that the increase in phenol contents during the grape drying raised their hydrophilic antioxidant activity. However, the correlation between the activity as linoleic acid/β-carotene reaction and the phenolic content was not significant, indicating that increased lipophilic antioxidant activity is not due to only the variation of phenolic compounds during the drying of Merlot and Tempranillo.

For all the above, it was more interesting to find a relationship between the hydrophilic antioxidant activity and the different fractions of phenolic compounds and only when making determinations *in vivo* based on a lipophilic balance such as that of β-carotene will be more interesting.[16] Also, the International

Table 2. A Simple Regression Analysis To Describe the Relationship between the Content in Phenolic Compounds and Hydrophilic Antioxidant Activity in the Two Grape Varieties

	correlation coefficient (r)	
	Merlot	Tempranillo
DPPH in Fraction 1		
Phenolic Acids		
gallic acid	0.8785	0.8680
protocatechuic acid	ns[a]	ns
p-OH-benzoic acid	0.9202	ns
vanillic acid	0.9597	0.9445
syringic acid	ns	0.8723
o-coumaric acid	ns	0.9482
p-coumaric acid	ns	ns
total	0.9461	0.9252
Esters		
c-caftaric acid	ns	0.8389
t-caftaric acid	ns	ns
c-coutaric acid	ns	−0.9174
t-coutaric acid	ns	−0.8444
c-feftaric acid	−0.9752	ns
t -feftaric acid	0.9081	0.9242
total	ns	ns
Flavans		
(+)-catechin	0.8752	0.8604
(−)-epicatechin	0.9632	0.9127
procyanidin B1	ns	ns
procyanidin B2 + B4	0.8681	0.9147
epigalocatequin gallate	ns	ns
epicatechin gallate	0.8091	0.8935
total	0.9674	0.8712
DPPH in Fraction 2		
Flavonols		
quercetin-3-glucoside	0.8823	ns
kaempferol-3-rutinoside	0.9247	ns
kaempferol-3-glucoside	0.9840	ns
myricetin	0.9647	ns
isorhamnetin-3-glucoside	0.9034	ns
quercetin	0.8723	0.9511
kaempferol	0.9395	0.9287
isorhamnetin	ns	0.9376
total	0.9215	ns
DPPH in Fraction 3		
Anthocyanin-3-glucoside		
delphinidin-3-glucoside	ns	ns
cyanidin-3-glucoside	ns	ns
petunidin-3-glucoside	ns	ns
peonidin-3-glucoside	ns	ns
malvidin-3-glucoside	ns	ns
total	ns	ns
Anthocyanin-3-acetylglucoside		
delphinidin-3-acetylglucoside	ns	0.9410
cyanidin-3-acetylglucoside	ns	0.9088
petunidin-3-acetylglucoside	ns	ns
peonidin-3-acetylglucoside	ns	ns
malvidin-3-acetylglucoside	ns	ns
total	ns	ns
Anthocyanin-3-coumaroylglucoside		
cyanidin-3-coumaroylglucoside	ns	0.8740
petunidin-3-coumaroylglucoside	ns	ns
peonidin-3-coumaroylglucoside	ns	0.8534
malvidin-3-coumaroylglucoside	ns	ns
total	ns	ns
Anthocyanin-3-caffeoylglucoside		
malvidin-3-caffeoylglucoside	ns	ns
total	ns	ns
Polymers		
polymers	0.9705	*0.8573*

[a] Not statistically significant at the 99.9% confidence level.

Organization of Vine and Wine endorses the DPPH assay as a fast, reliable tool for assessing antioxidant activity in grapes and wine.[33] Based on the foregoing, we measured hydrophilic antioxidant activity (DPPH) in three different phenol fractions obtained by fractionation. Fraction 1 contained the combination of phenolic acids, esters and flavans; fraction 2 contained flavonols, and fraction 3 anthocyans and polymers. As shown by some authors, not all fractions are identically correlated since not all phenol compounds possess the same antioxidant activity.[10,11,16]

Figure 3 shows the antioxidant activity of fraction 1 from Merlot and Tempranillo grapes as measured with the DPPH assays, as well as the combined concentration of the phenolic compounds present in this fraction. As can be seen, an increase in contents in the phenolic compounds of this fraction (from 37.7 to 94.1 mg/L in Merlot and 85.8 to 147 mg/L in Tempranillo) resulted in an increase in hydrophilic antioxidant activity (from 0.16 to 0.59 mmol TE/L in Merlot and 0.22 to 1.56 mmol TE/L in Tempranillo). In addition, the increased concentration of phenols in Tempranillo must caused an increased antioxidant activity in these musts. A simple regression analysis (Table 2) revealed the absence of significant correlations with hydroxycinnamic esters and a significance of 99.9% ($p < 0.001$) for the phenolic acid (r = 0.9461 in Merlot and r = 0.9252 in Tempranillo) and flavans (r = 0.9674 in Merlot and r = 0.8712 in Tempranillo) fractions.

Specifically, regression against the different compounds revealed that gallic and vanillic were the phenolic acids exhibiting the highest significance in the two grapes varieties. Except that of *t*-feftaric acid, none of the hydroxycinnamic esters exhibited correlation between its concentration and antioxidant activity in the two grape varieties; by contrast, all flavans except epigallocatechin gallate and procyandin B1 exhibited such correlation. Tabart et al.[14] found hydrophilic antioxidant activity in grape skins to be correlated with the total flavan contents.

Figure 4 shows the hydrophilic antioxidant activity of fraction 2, and the combined concentration of its components (flavonols). As can be seen, an increase in contents in the phenolic compounds of this fraction in Merlot (from 3.83 to 14.2 mg/L) resulted in a gradual increase in antioxidant activity (from

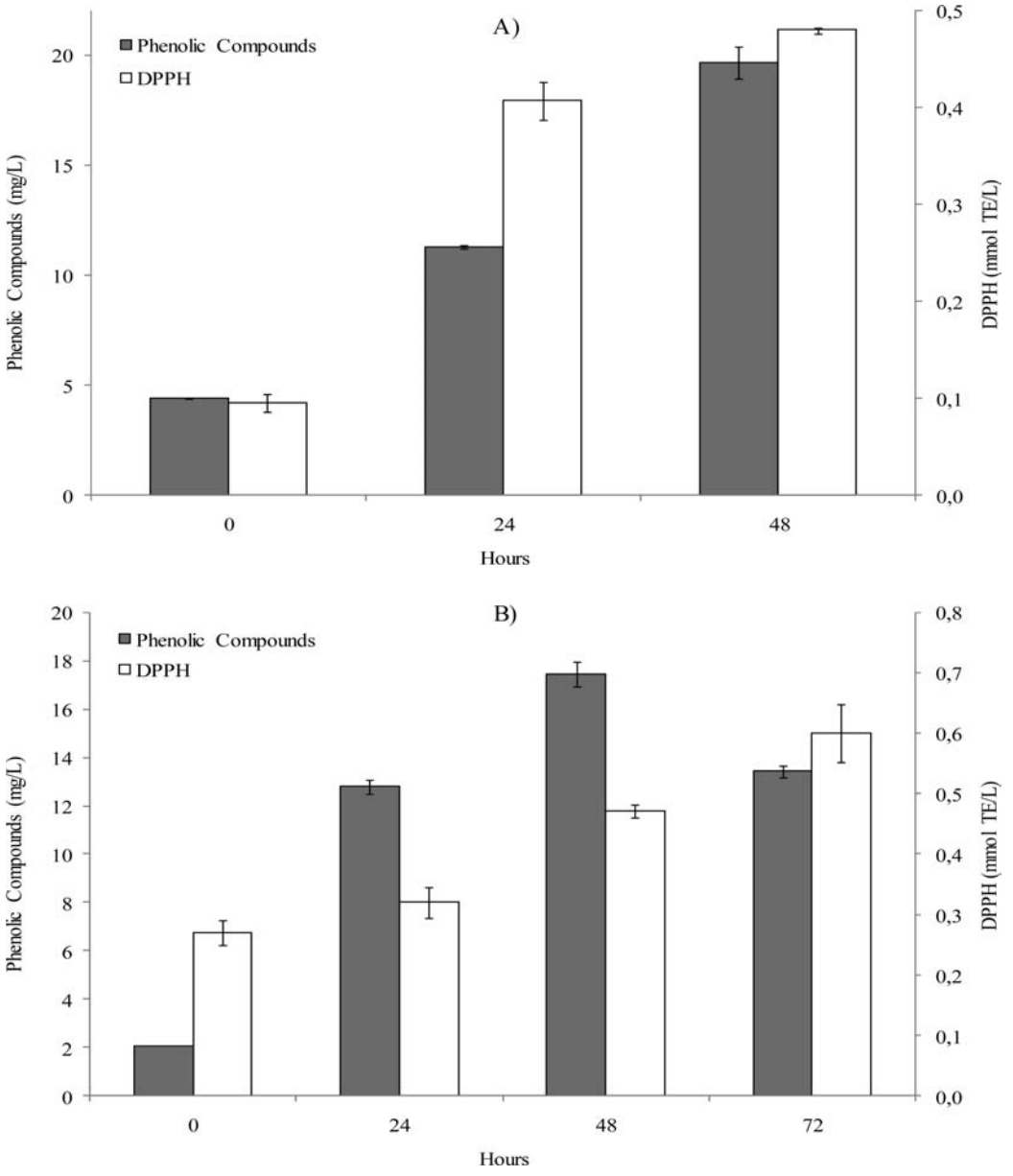

Figure 4. Antioxidant activity measured with the DPPH assays and combined concentration of the phenol compounds of fraction 2 from Merlot (A) and Tempranillo (B) grapes.

0.10 to 0.48 mmol TE/L). The increase was significant at the 99.9% level ($r = 0.9215$). On the other hand, the variation of the contents in flavonols in Tempranillo (a rise over the first 48 h and a subsequent fall) was accompanied by no gradual increase in antioxidant activity, which changed from 0.2 to 0.6 mmol TE/L. This suggests that changes in some flavonols in this variety were not significant. This was confirmed by correlating the activity of fraction 2 with the concentration of each compound in it (Table 2). In fact, while all flavonols except isorhamnetin were significantly correlated in Merlot, only aglucones were in Tempranillo. Brenna et al.[34] found a good correlation between antioxidant activity and the quercetin and myricetin contents of red wines. On the other hand, Fernandez-Pachon et al.[35] concluded that this phenol fraction plays no prominent role in antioxidant activity.

Figure 5 shows the variation of the hydrophilic antioxidant activity of the anthocyan and polymer fraction, as well as their combined concentrations. Note that this fraction accounted for more than 50% of the total antioxidant activity in both grape varieties. This suggests that anthocyans and phenol polymers contribute greatly to this property. Katalinic et al.[1] previously found a significant correlation between hydrophilic antioxidant activity as measured with the DPPH assay and the total anthocyan contents of red grapes, and so did in red grape skins.[14]

During drying, the concentration of the compounds in fraction 3 increased from 83.2 to 484 in the Merlot variety and 144 to 604 mg/L in the Tempranillo variety. This increase caused an increase in the antioxidant activity from 2.15 to 6.83 mmol TE/L in the former and 3.04 to 6.62 mmol TE/L in the latter. The results of a regression analysis (Table 2) revealed that phenolic polymers were the only compounds significantly correlated with the antioxidant activity in Merlot. In Tempranillo so were the acyl derivatives of delphinidin and cyanidin, coumaroyl derivatives of cyanidin and peonidin, and the polymers identified

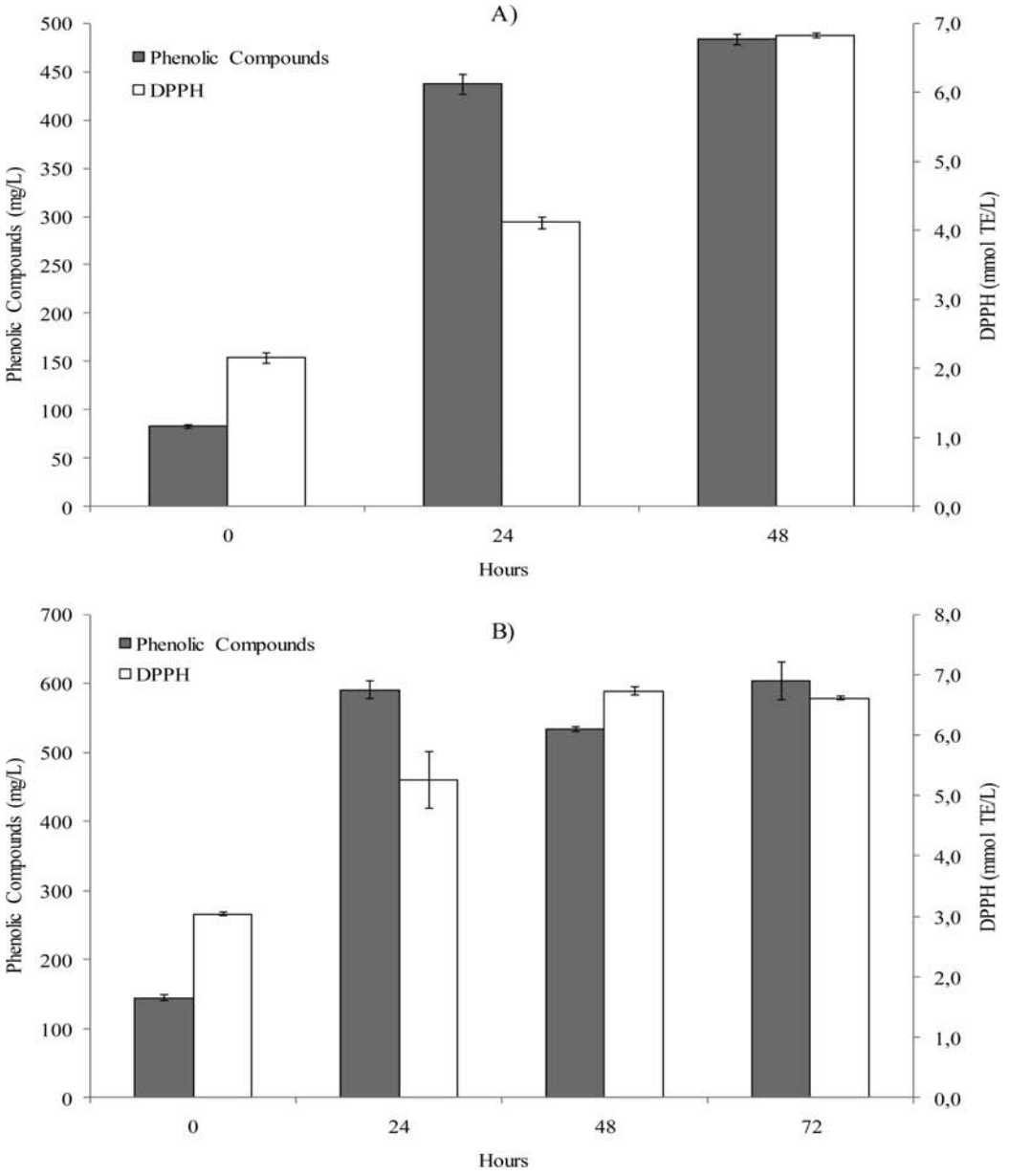

Figure 5. Antioxidant activity measured with the DPPH assays and combined concentration of the phenol compounds of fraction 3 from Merlot (A) and Tempranillo (B) grapes.

by HPLC. Alen-Ruiz et al.[16] only found acyl derivatives to exhibit the correlation with antioxidant activity in three month old red wines.

In summary, Merlot and Tempranillo red grapes dried under controlled temperature and moisture conditions in a chamber with a view to the production of sweet wines exhibit a typical phenolic profile where anthocyanins prevail, and flavonols and phenolic acids are present in lower proportions than flavans. Drying in the chamber raised the concentrations of all phenol compounds except the esters, which increased by the effect of water evaporation, extraction from grape skins or a chemical reaction of hydrolysis and/or biosynthesis. Drying also increased hydrophilic and lipophilic antioxidant activities, which are based on electron- and proton-transfer reactions, respectively. Hydrophilic activity was more closely related to the increase in phenol compounds than was lipophilic activity. Specifically, the hydrophilic activity was seemingly due to a great extent to polymers in addition to phenolic acids, flavans and individual flavonols and anthocyans. Therefore, chamber drying, which is economical and reduces the risk of fungal synthesis of ochratoxin A, boosts the antioxidant activity of grapes.

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