ANTHOCYANIN COMPOSITION AND EXTRACTION FROM GRENACHE NOIR (*Vitis vinifera* L.) VINE LEAF USING AN EXPERIMENTAL DESIGN I- BY ETHANOL OR SULFUR DIOXIDE

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Abstract

Aim: Anthocyanins are water soluble pigments mainly located in grape skin; however, these phenolic compounds are also located in vine leaves. The aim of this work was to identify, quantify and determine for the first time the anthocyanin composition in Grenache noir (*Vitis vinifera*) leaves.

Methods and results: Five anthocyanins were identified and quantified in Grenache noir leaves by HPLC-UV-MS. Of these, cyanidin-3-*O*-glucoside and peonidin-3-*O*-glucoside were the main anthocyanins and represented 44 and 37%, respectively, while delphinidin-3-*O*-glucoside, petunidin-3-*O*-glucoside and malvidin-3-*O*-glucoside accounted for only 6, 5 and 8%, respectively. A multi-factorial design experiment was used to build a mathematical model to estimate the best extraction condition (highest anthocyanin extraction yield from leaf extract) for both SO_2 and ethanol in the aqueous extraction solvent. For SO_2, the optimal extraction parameters were an extraction time ranging between 5.77 and 6 h, a temperature ranging between 20 and 23.7°C and a concentration of 500 ppm of SO_2 in the aqueous extraction solvent. Using the hydro-alcoholic extraction solvent, the optimal extraction parameters were an extraction time ranging between 3 and 4.37 h, a temperature set at 20°C and an ethanol concentration in the extraction solvent ranging between 32 and 40%.

Conclusion: Only the five mono-glucoside anthocyanins usually detected in grapes have been detected, indentified and quantified in Grenache noir leaves by HPLC-UV-MS. Using a multi-factorial design experiment the optimum conditions for the extraction of these anthocyanins were obtained in hydro-alcoholic solution (i.e., extraction time ranging between 3 and 4.37 h, a temperature set at 20°C and an ethanol concentration ranging between 32 and 40%) and in water with SO_2 (i.e., extraction time ranging between 5.77 and 6 h, a temperature ranging between 20 and 23.7°C and a concentration of 500 ppm of SO_2 in water).

Significance and impact of the study: Five anthocyanins were detected, identified and quantified in Grenache noir leaves by HPLC-UV-MS and the main anthocyanin were with cyanidin-3-*O*-glucoside and peonidin-3-*O*-glucoside. The optimum conditions for the extraction of these anthocyanins were estimated in hydro-alcoholic solution and in water solvent with SO_2 which will allow further investigation on these anthocyanins to be used as natural pigment for the food as well as for the pharmaceutical and cosmetic industry.

Key words: anthocyanins, leaves, Grenache noir, *Vitis vinifera* L., extraction, factorial design

Résumé

Objectif : Les anthocyanes sont des pigments solubles dans l'eau qui sont principalement localisées dans la pellicule des raisins de Grenache noir, cependant ces composés phénoliques sont également présents dans les feuilles. Le but de ce travail a été d'identifier, quantifier et déterminer pour la première fois la composition des anthocyanes présentes dans les feuilles de Grenache noir (*Vitis vinifera*).

Méthodes et résultats : Cinq anthocyanes ont été identifiées et quantifiées dans des feuilles de Grenache noir par HPLC-UV-MS. Parmi ces anthocyanes, la cyanidine-3-*O*-glucoside et la péonidine-3-*O*-glucoside étaient les anthocyanes majoritaires représentant respectivement 44 et 37%, alors que la delphinidine-3-*O*-glucoside, la pétunidine-3-*O*-glucoside et la malvidine-3-*O*-glucoside ne représentaient que 6, 5 et 8% respectivement. Le plan d'expérience multifactoriel a été utilisé pour construire un modèle mathématique permettant d'estimer le meilleur rendement d'extraction d'anthocyanes qui sera obtenu pour un temps d'extraction entre 5.77 et 6 h, une température comprise entre 20 et 23.7°C et une concentration de SO_2 dans l'eau de 500 ppm. En outre, une étude similaire en utilisant un solvant d'extraction hydro-alcoolique a révélé que les paramètres optimaux d'extraction des anthocyanes sont obtenus pour un temps d'extraction compris entre 3 et 4.37 h, une température fixée à 20°C et une concentration en éthanol dans le solvant d'extraction comprise entre 32 et 40%.

Conclusion: Uniquement les 5 anthocyanes mono-glucoside généralement identifées dans le raisins ont été détectées, identifiées et quantifiées par HPLC-UV-MS dans des feuilles de Grenache noir. L'utilisation d'un plan d'expérience multifactoriel a permis de déterminer les conditions d'extraction optimum de ces anthocyanes en solution hydro-alcoolique (temps d'extration entre 3 et 4.37 h, une température de 20°C et concentration en éthanol entre 32 et 40%) une ainsi qu'en solution aqueuse contenant du SO_2 (temps d'extration entre 5.77 et 6 h, une température entre 20 et 23.7°C et concentration de SO_2 de 500 ppm dans l'eau).

Signification et impact de l'étude: Les cinq anthocyanes détectées, identifiées et quantifiées dans des feuilles de Grenache noir sont les anthocyanes mono-glucosides généralement également présentes dans le raisin. Les conditions d'extraction optimum de ces anthocyanes ont été estimées en solution hydro-alcoolique ainsi qu'en solution aqueuse contenant du SO_2 ce qui va permettre d'étudier l'utilisation potentiel de ces anthocyanes comme colorant naturels alimentaires ou pour l'industrie pharmaceutique et cosmétique.

Mots clés : anthocyanes, feuilles, Grenache noir, *Vitis vinifera* L., extraction, plan d'expérience factoriel

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INTRODUCTION

Anthocyanins are water soluble pigments that are widespread in the plant kingdom, especially in angiosperms and flowering plants (Mazza and Miniati, 1993 ; Bridle and Timberlake, 1997). They are polyphenolic compounds belonging to the flavonoid family and are responsible for the red and blue color of plants (McDougall *et al.*, 2005). Anthocyanin molecules are composed of an anthocyanidin backbone (also called flavylium ion moieties) on which different types of sugar moieties are glycosylated rather than esterified. The structures of the anthocyanidins differ in their degree of hydroxylation and degree of methoxylation, and this difference results in color modulation ranging from red-orange shades for pelargonidin to violet-blue for delphinidin at pH 1 (Stintzing and Carle, 2004). In general, hydroxylation of the anthocyanin B-ring induces a bathochromic shift, while the methylation of a B-ring hydroxyl group results in an opposite effect (Brouillard and Delaporte, 1977). In grape berries from *Vitis vinifera* L. varieties, there are fifteen different anthocyanins divided into three groups depending on the glucosidic group in position 3 (i.e., mono-glucoside, acethyl-glucoside and *p*-coumaroyl-glucoside). There are five different anthocyanidins in each group based on the aromatic B-ring substitutions [i.e., malvidin (Mv), delphinidin (Dp), peonidin (Pn), petunidin (Pt) and cyanidin (Cy)] (Glories, 1984 ; Hebrero *et al.*, 1988). Moreover, the profile and concentration of anthocyanins in red grapes change according to species, variety, maturity, seasonal conditions, production areas, the type/style of wine to be produced and management practices (Esteban *et al.*, 2001 ; Kelebek *et al.*, 2010). However, the anthocyanin composition in vine leaves from *Vitis vinifera* L. varieties is poorly known. To our knowledge, only two studies have been reported for Carignan (Ezzili *et al.*, 1999), Merlot and Cabernet sauvignon (Darné and Glories, 1988), revealing that only the five monoglucoside anthocyanins were observed in vine leaves at leaf fall (Ezzili, 2001).

Anthocyanin pigments are well known as natural red colorants in foods (Kırca *et al.*, 2007), making them important for the food industry (McDougall *et al.*, 2005). These natural colorants arouse increasing interest due to their wide range of colors (i.e., deep red to deep blue), their high solubility in aqueous media, as well as their effects on health (Teszlak *et al.*, 2005). Anthocyanins have been extracted from various plant sources and by-product, particularly from grape skins and pomace, to produce pigment for the food, cosmetic and medical industry. However, only the two previously cited studies have focused on vine leaf anthocyanins. Moreover, the anthocyanins deriving from wine and pomace are well known for their antioxidant capabilities (Rivero-Pérez *et al.*, 2008 ; Corrales *et al.*, 2009 ; Kırca *et al.*, 2007), as well as for a large variety of other biological activities. Among others, they exhibit vitamin P activities (Jonadet *et al.*, 1983), antioxidant activities (Teissedre *et al.*, 1996) and protective activities against coronary heart disease (Gülçin *et al.*, 2005). Moreover, some veinotonic effect and anti-edematous action have also been observed (Kiesewetter, 2002 ; Schaefer *et al.*, 2003).

Despite the great potential of anthocyanins as natural pigment for the food as well as for the pharmaceutical and cosmetic industry, their use has been limited due to their relative instability and their low percentage of extraction. Currently, all the investigations on anthocyanins focus on solving these problems as well as on purifying and identifying new anthocyanins in plants (Castañeda-Ovando *et al.*, 2009). Solvents such as ethanol, methanol or sulfur dioxide (SO_2) in water are generally used for the extraction of natural pigments. Of these, methanol is not privileged by the food industry in order to prevent the toxicity of the final product. Similarly, SO_2 in water can present health risks even at low concentrations (Spagna *et al.*, 2003). Compared to all solvents, anthocyanin extraction by ethanol is the most acceptable solvents for the food industry (Li *et al.*, 2006). The extraction of anthocyanins from the skins of red grapes has been described by several methods (Sriram *et al.*, 1999), and the influence of several parameters such as time, pressure, light intensity (Corrales *et al.*, 2009), solvent concentration (Mazza and Miniati, 1993) and temperature (Cacace and Mazza, 2002) on the extraction yield has been evaluated.

However, it appears that the extraction of anthocyanins from vine leaves has not been studied. Therefore, the aim of this work was to evaluate the influence of temperature, time and solvent composition (i.e., hydro-alcoholic or SO_2 in water) on the anthocyanin extraction yield from Grenache noir leaves. The experimental factorial design method was used to design this study. Along with time and temperature, we also focused on the impact of ethanol and SO_2 on the extraction rate of anthocyanins. Ethanol was chosen since it is an efficient solvent for anthocyanidin extraction (Patil *et al.*, 2009). On the other hand, even if there are some health risks associated with SO_2, it is well known to increase the yield of anthocyanin extraction (Bridle and Timberlake, 1997 ; Ayed *et al.*, 2000). The experimental factorial design method will help to meet our requirements. Indeed, its principle is to simultaneously vary the levels of one or more factors for each test in order to reduce the number of experiments and to increase the number of studied factors. Moreover, such method allows the detection of interactions between factors as well as the optimal conditions. The main advantage of using the experimental factorial design is to minimize as much as possible the number of experiments needed without sacrificing the accuracy of the results.

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MATERIALS AND METHODS

1. Chemicals

Formic acid (>95%) and hydrochloric acid (37 %) were purchased from Sigma-Aldrich (St Quentin Fallavier, France). Milli-Q (Millipore) water was prepared using a Sartorius-arium 611 system. HPLC-grade ethanol and acetonitrile were obtained from Merck (Darmstadt, Germany). Anthocyanin standards, delphinidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, peonidin-3-*O*-glucoside and malvidin-3-*O*-glucoside, were purchased from Extrasynthese (Genay, France).

2. Plant material

The study was conducted on *Vitis vinifera* L. cv. Grenache noir grafted on Richter 99, which resulted from a massal selection in Tunisia, and planted in the Bir Bouregba area, Nabeul (Tunisia) (10° 25' latitude and 36° 37W longitude ; 20 meters above sea level). In the vineyard, the vines were planted at 2 m (vine spacing) x 3 m (row spacing). Standard fertilization and treatments were performed as for commercial vineyard. The vine leaves at the opposite side of the first cluster were collected on 8th December, 2009. After collection, the leaves were dried at 20 °C in a dark room and crushed in a Forplex crusher. The obtained powder was stored in the dark at 20 °C until needed.

3. Extraction process

0.5 g of vine leaf powders were extracted in the dark with 50 mL of the extraction solvent as specified in table 1. The factors considered were the extraction time (h), the extraction temperature (°C) and the solvent concentration. Prior to the anthocyanin identification by HPLC-UV and quantification by spectrophotometry coupled to HPLC-UV, all the extraction mixtures were filtered through a 0.45 µm filter membrane.

4. Determination of anthocyanin concentration

The determination of the total anthocyanin concentration (TAC) was performed by spectrophotometry using the pH differential method (Giusti and Wrolstad, 2001 ; Wrolstad *et al.*, 2005). Each sample obtained after extraction was divided into two aliquots diluted with the corresponding buffer solutions at pH 1 and pH 4.5 (i.e., 600 µL of sample in 2.4 mL buffer solution). Such fifth dilution preserves the effect of buffer and stays within the range of reliable absorbance spectrophotometer (DO < 1.5). The absorbances were

Table 1 - Experimental matrix of complete factorial design 2^3.

Tests	Factors			
	Time (h)	Temperature (°C)	Solvent composition	
			[EtOH] (%)	[SO_2] (ppm)
1	3	20	20	/
2	6	20	20	/
3	3	40	20	/
4	6	40	20	/
5	3	20	40	/
6	6	20	40	/
7	3	40	40	/
8	6	40	40	/
1	3	20	/	500
2	6	20	/	500
3	3	40	/	500
4	6	40	/	500
5	3	20	/	2000
6	6	20	/	2000
7	3	40	/	2000
8	6	40	/	2000

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measured 20 min after dilution in order to insure complete equilibrium between all the anthocyanin forms at 520 nm and 700 nm. TAC in each sample was calculated using the formula below and expressed in mg of anthocyanins per g of leaf powder dry weight (dw).

$$TAC\ (mg.L^{-1}) = (A \times M_W \times DF \times 1000) / (\varepsilon \times 1) \quad (1)$$

Where: A: calculated as $(A_{520nm} - A_{700nm})\ pH_{1.0} - (A_{520nm} - A_{700nm})\ pH_{4.5}$
M_W: molecular weight of cyanidin-3-*O*-glucoside
DF: dilution factor
l: optical way (i.e., 1 cm)
ε: 20653 $l.mol^{-1}.cm^{-1}$ the molar extinction coefficient of cyanidin-3-*O*-glucoside estimated as described below.

5. Determination of the molar extinction coefficient of cyanidin-3-*O*-glucoside

Six acidified aqueous solutions (i.e., 1 % HCl) of cyanidin-3-*O*-glucoside standard (i.e., 2.5, 5, 10, 25 and 50 $mg.L^{-1}$) were realized starting from an initial solution of 200 $mg.L^{-1}$. The molar extinction coefficient of cyanidin-3-*O*-glucoside was established by plotting the obtained absorbance at 520 nm versus the concentration of the standard using the law of Beer-Lambert (DO =εlC).

6. Determination of the anthocyanin composition in leaves by HPLC-UV

0.5 g of leaf powder were extracted with 50 mL of the solvent specified in table I at 20 °C for 3 h. The obtained aqueous extract was then filtered through a 0.45 µm filter membrane and evaporated to dryness under vacuum at 30 °C. The obtain residue was re-dissolved in 5 mL of acidified water (i.e., 1 % formic acid), filtered through a 0.45 µm filter membrane, and injected in HPLC-UV to estimate the anthocyanin composition of the leaf extract.

These HPLC-UV analyses were performed on a Beckman system composed of a PDA detector (Diode Array Detector 168), an autosampler and a quaternary pump system (System Gold HPLC 126) and controlled by New Gold software. These analyses were carried out in duplicate on a 250 x 4.6 mm i.d. 4 µm Synergy RP-Max column (Phenomenex). The mobile phase was composed of solvent A [H_2O – HCOOH (95:5)] and solvent B [acetonitrile – HCOOH (95:5)] with a gradient elution (0 – 3 min, 3 % solvent B; 3 – 20 min, 3 % to 10 % solvent B; 20 – 30 min, 10 % to 20 % solvent B; 30 – 45 min, 20 % to 25 % solvent B; 45 – 60 min, 25 % to 55 % solvent B; 60 – 62 min, 55 % to 100 % solvent B; 62 – 70 min, 100 % solvent B) applied at a flow rate of 1 $mL.min^{-1}$ with detection set at 520 nm. The anthocyanins were identified and assigned by comparison of their retention time and UV spectra with authentic standards.

7. Experimental design

A complete factorial design was applied to identify the optimal conditions of anthocyanin extraction from the vine leaf. The effects of three independent variables (i.e., solvent composition, extraction temperature and time) on the anthocyanin concentration were investigated. The experimental matrix (Table 1) was determined from the software MINITAB 14.0.

RESULTS AND DISCUSSION

1. Anthocyanin composition in Grenache noir leaf extracts

In the extracts obtained from Grenache noir vine leaves, only five different anthocyanins were identified by HPLC-UV-MS according to their retention time, elution order and spectral characteristics (i.e., mass and UV-Vis spectrum). These anthocyanins were assigned to delphinidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, peonidin-3-*O*-glucoside and malvidin-3-*O*-glucoside (Kelebek *et al.*, 2010). Among these, cyanidin-3-*O*-glucoside and peonidin-3-*O*-glucoside were the main anthocyanins since they accounted for 44 and 37 % of all the anthocyanins, respectively. Delphinidin-3-*O*-glucoside, petunidin-3-*O*-glucoside and malvidin-3-*O*-glucoside accounted for only 6, 5 and 8 %, respectively (Figure 1). Such anthocyanin composition is drastically different from the anthocyanin composition generally observed in grape or wine in which malvidin-3-*O*-glucoside is the main anthocyanin (Kelebek *et al.*, 2006). However, such anthocyanin composition in vine leaf has been previously observed and reported in Carignan (Ezzili *et al.*, 1999), Merlot and Cabernet sauvignon (Darné and Glories, 1988) leaves at leaf fall. Furthermore, the extraction conditions (i.e., time, temperature and SO_2 concentration or ethanol percentage in the extraction solvent) did not have a significant effect on the extracted anthocyanin compositions since the ratios between each anthocyanin remained similar (Figure 1).

2. Determination of the optimum anthocyanin extraction conditions using SO_2

The impact of each factor (i.e., time, temperature, SO_2 concentration) on the TAC was estimated by comparing the curves of the average response for each studied factor using the MINITAB 14.0 software (Figure 2A). The main effects of the three factors taken separately revealed that an increase of both time and temperature have a positive effect on the extracted TAC, while the increment of SO_2 concentration from 500 ppm to 2000

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ppm in the extraction solvent has a negative effect. This last factor had an important influence on the response since the slope of the curve dramatically decreased compared to the other two curves. When the SO_2 concentration increased from 500 ppm to 2000 ppm in the extraction solvent, the TAC decreased by about 10 % (i.e., from 7.15 $mg.g^{-1}$ dw to 6.5 $mg.g^{-1}$ dw). Although it has been previously observed that SO_2 increases anthocyanin extraction yield by improving the dissemination of anthocyanins through cell walls (Gao and Mazza, 1996), here the strong negative impact of 2000 ppm SO_2 on the yield of anthocyanin extraction likely results from a reaction between SO_2 and anthocyanins leading to the formation of colorless compounds.In order to estimate the best extraction condition, the interaction between each factor was evaluated by looking at the response curve of opposite factors compared in pairs (Figure 2B). The resultant interaction curves showed that the 3 h extraction time has a positive effect on the anthocyanin extraction yield when coupled with the highest extraction temperature (Figure 2B-panel 1) and a negative effect with increasing SO_2 concentration (Figure 2B-panel 2), whereas the 6-h extraction time has a negative effect with high temperature and high SO_2 concentration (Figure 2B-panel 2). Similar effects were observed for SO_2 concentration with low and high temperature (Figure 2B-panel 3). Regarding temperature, a positive effect on the anthocyanin extraction yield of 20 °C and a negative effect of 40 °C with time were noticed (Figure 2B-panel 1). The observed negative impact of an extraction temperature set at 40 °C compare to 20 °C on the anthocyanin extraction yield was most likely a result of the thermal degradation of the extracted anthocyanins.

Moreover, the optimum condition for anthocyanin extraction using SO_2 was confirmed using the multi-factorial design experiments 2^3. The TAC values obtained for each experimental test and the weighting coefficients (Wc) of each factor and possible interaction between them are reported in table 2. These data showed that time and temperature have a positive effect (0.022 and 0.089, respectively) on the TAC extracted. By contrast, SO_2 concentration had a significant negative effect (-0.662). Regarding the interaction between the factors, the time-temperature interaction and the time-[SO_2] interaction had a negative effect (-0.159 and -0.116, respectively). However, the temperature-[SO_2] interaction and the interaction between the three factors had a positive effect on the TAC extracted (0.009 and 0.038, respectively). Overall, TAC was the highest (7.27 $mg.g^{-1}$ dw) with the test 2 condition, while the lowest value was obtained with test 5 (6.42 $mg.g^{-1}$ dw).

Finally, by plotting the 2D contour curve on the studied intervals for each factor, it was possible to determine the optimum conditions for anthocyanin extraction. Thus, TAC would be the highest for an extraction time ranging between 5.77 and 6 h, a temperature set between 20 and 23.7°C and 500 ppm of SO_2 in the extraction solvent (Figure 3A). Moreover, from this 2D contour curve, it was possible to obtain the polynomial equation below taking into account the three studied factors (i.e., time, temperature, SO_2 concentration) to estimate the anthocyanin extraction yield.

$$[TAC] = 7.2 + 0.0075\,t + 0.00445\,T - 0.000441\,[SO_2] \quad (R^2 = 91.7\ \%)$$

With: - [SO_2]: concentration of sulfur dioxide (in ppm)
- T: Temperature (°C)
- t: time (h)

The strength of the mathematical model was confirmed by the coefficient of determination $R^2 = 91.7$ %,

Table 2 - Weighting coefficients (Wc) of the main factors and interactions.

Factor	1	2	3	Effect 1*2	Effect 1*3	Effect 2*3	Effect 1*2*3	[TAC]
Test	Time	Temp	[SO_2]					($mg.g^{-1}$ dw)
1	-	-	-	+	+	+	-	6.93
2	+	-	-	-	-	+	+	7.27
3	-	+	-	-	+	-	+	7.21
4	+	+	-	+	-	-	-	7.15
5	-	-	+	+	-	-	+	6.42
6	+	-	+	-	+	-	-	6.44
7	-	+	+	-	-	+	-	6.63
8	+	+	+	+	+	+	+	6.42
Wc	0.022	0.089	-0.662	-0.159	-0.116	0.009	0.038	/

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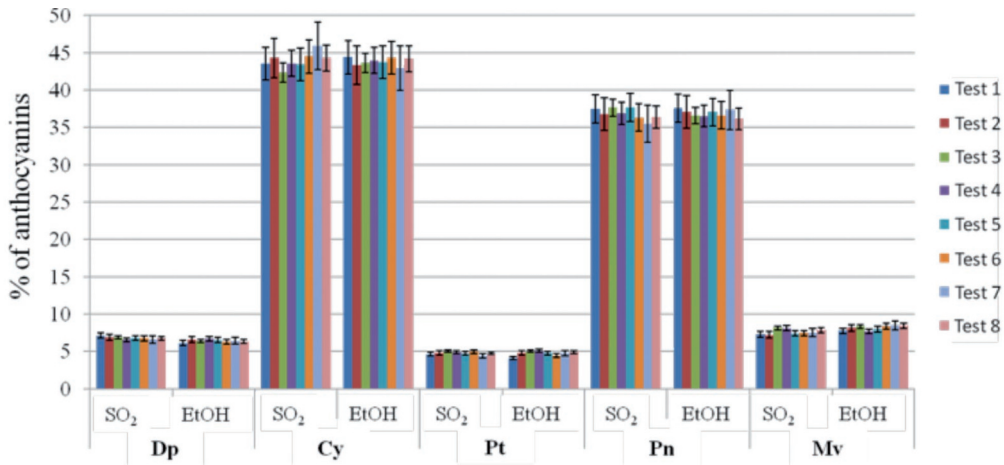


Figure 1 - Comparison of anthocyanin composition in Grenache noir vine leaves estimated by HPLC-UV for the different extraction conditions. For each monomeric anthocyanin and solvent, the extraction condition tests 1 to 8 are plotted from left to right. Dp: delphinidin-3-*O*-glucoside, Cy: cyanidin-3-*O*-glucoside, Pt: petunidin-3-*O*-glucoside, Pn: peonidin-3-*O*-glucoside, Mv: malvidin-3-*O*-glucoside.

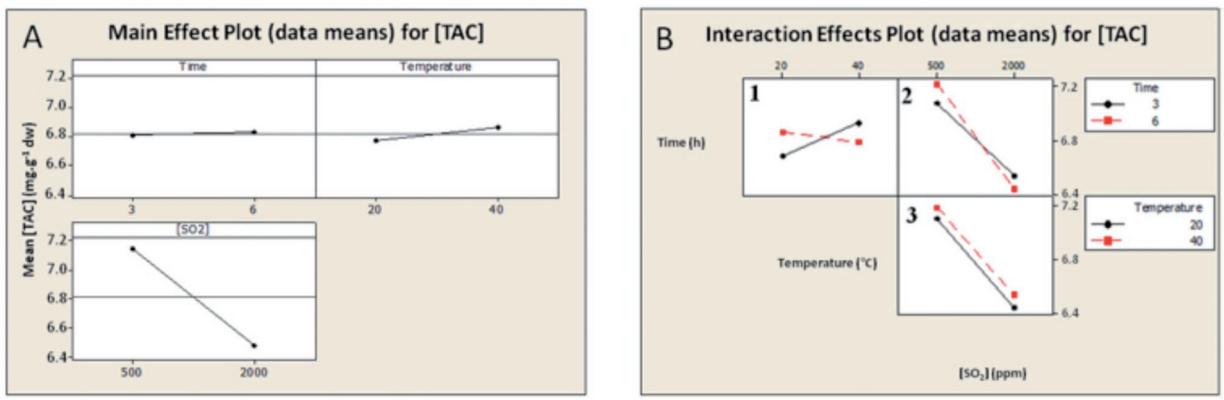


Figure 2 - A: Effects of the main factors on the total anthocyanin concentration ([TAC]) response; B: Interactions between factors as a function of TAC, with (1) Time/Temperature Interaction, (2) Time/[SO_2] Interaction; and (3) Temperature/[SO_2] Interaction. [TAC] is expressed as mg.g^{-1} dry leaf extract.

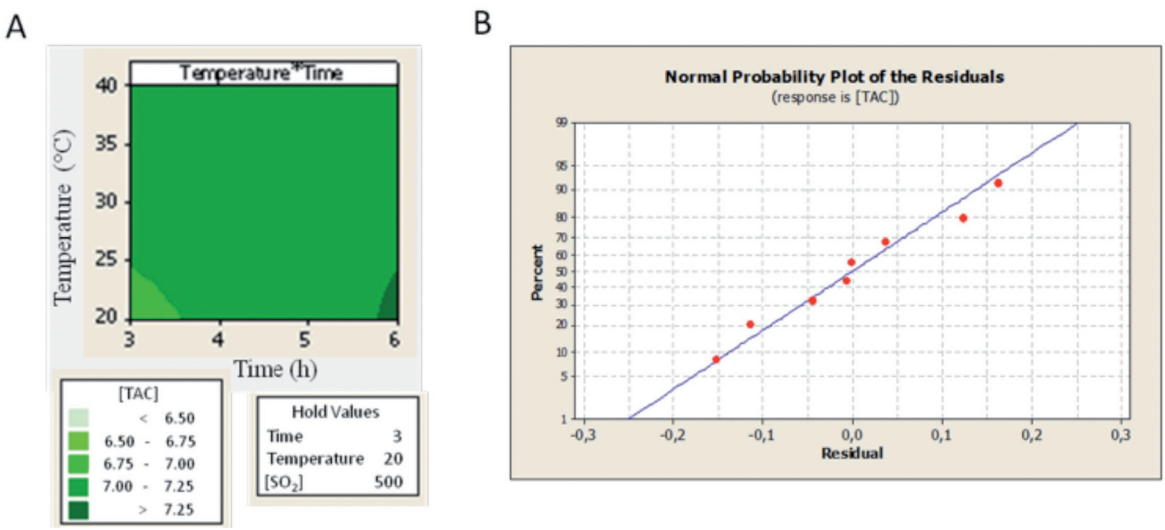


Figure 3 - A: Contour curve for total anthocyanin concentration ([TAC]) based on temperature and time at a constant [SO_2] (500 ppm); B: Normal probability plot of the mathematical model.

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which was statistically significant at p = 0.013. Moreover, figure 3B shows the variation of the residual values and the normal probability that was used to confirmed that the model corresponds to the normal probability curve. According to this figure, it is possible to affirm that the model is adequate, because in the opposite case, the points would have been scattered in the graphic space rather than show a normal probability distribution.

Moreover, according to the mathematical model, the highest anthocyanin extraction yield would be obtained for an extraction time ranging between 5.77 and 6 h, a temperature ranging between 20 and 23.7°C and a SO_2 concentration in water of 500 ppm.

In all, these results have shown that SO_2 can be added to anthocyanin extraction solvent in order to improve the yield of extraction, although at low level (i.e., 500 ppm). This is in agreement with the data reported by Bridle and Timberlake (1997) and Ayed *et al.* (2000). The addition of SO_2 in the extraction solvent leads to the formation of a complex between SO_2 and anthocyanins, which facilitates the dissociation of the interaction between anthocyanins and some macromolecules such as pectin, cellulose and proteins. Gao and Mazza (1996) suppose that SO_2 is improving the dissemination of anthocyanins through cell walls, which increases the solubility of the pigments. SO_2 also plays the role of protecting agent against anthocyanin oxidation (Gao and Mazza, 1996).

3. Determination of the optimum anthocyanin extraction conditions using ethanol

Following the above study regarding the impact of SO_2 concentration in the extraction solvent on the anthocyanin extraction yield from Grenache noir leaves, the influence of ethanol level together with time and temperature was then evaluated. Using a similar pattern, the impact of each factor (i.e., time, temperature, ethanol concentration) on TAC was estimated by comparison of the average response curves for each studied factor (Figure 4A). The main effects of the three factors taken separately showed that both the time and temperature factors have a negative or lower effect on the anthocyanin extraction yield compare to the important positive impact of the ethanol content in the extraction solvent. This large influence of ethanol on the anthocyanin extraction yield is revealed by the slope of the curve reported in figure 4A. It is therefore possible to estimate that using a concentration of 40 % ethanol and a temperature set at 20 °C for 3 h will maximize the anthocyanin extraction yield.

The interactions between each factor were evaluated by looking at the response curve of opposite factors compared in pairs (Figure 4B). The resultant interaction curves showed that the 6-h extraction time has a negative effect when coupled to the highest extraction temperature (Figure 4B-panel 1) and a positive effect with the increase of ethanol concentration (Figure 4B-panel 2). Moreover, concerning temperature, it was noted a negative effect of the highest extraction temperature when coupled to the increase of the extraction time (Figure 4B-panel 1) as well as to the ethanol concentration (Figure 4B-panel 3).

The optimum condition for anthocyanin extraction using ethanol was confirmed using the multi-factorial design experiments 2^3. The TAC values obtained for each experimental test and the Wc of each factor and possible interactions between them are reported in table 3. The Wc for time and temperature (-0.29 and -0.654, respectively) revealed that these two factors have a negative effect on the anthocyanin extraction yield. By contrast, the ethanol concentration in the extraction solvent had a significant positive effect on the anthocyanin extraction yield (0.408). The comparison of the interaction between factors revealed that the time-temperature, time-ethanol concentration and temperature-ethanol concentration interactions have a positive effect on TAC, as shown by their respective Wc (0.036, 0.034 and 0.030). However, the interaction between the three factors had a negative effect on the anthocyanin extraction yield (-0.042). The highest extracted anthocyanin concentration was observed with the test condition 5 (6.12 $mg.g^{-1}$ dw; 3 h, 20 °C and 40 % ethanol), whereas the test condition 4 extracted the lowest amount of anthocyanins (4.80 $mg.g^{-1}$ dw; 6 h, 40 °C, 20 % ethanol). As previously observed, the strong negative influence of temperature on the amount of extracted anthocyanins might be due to the thermal degradation of the extracted anthocyanins. Similarly, the negative impact of time results from the same mechanism since more extracted anthocyanins will be degraded with longer extraction time.

Finally, by plotting the 2D contour curve on the studied intervals for each factor, it was possible to estimate the optimum conditions for anthocyanin extraction. Thus, the TAC would be highest (i.e., above 6 $mg.g^{-1}$ dw) for an extraction time ranging between 3 and 4.37 h, a temperature set at 20 °C and an ethanol concentration in the extraction solvent ranging between 32 and 40 %, as depicted in figure 5A. Moreover, from this 2D contour curve, it was possible to obtain a polynomial equation taking into account the three studied factors (i.e., time, temperature and ethanol concentration) to estimate the best anthocyanin extraction yield.

$$[TAC] = 6.28 - 0.096\,t - 0.032\,T + 0.02\,[EtOH] \quad (R^2 = 99.2\ \%)$$

With: - [EtOH]: ethanol concentration in the solvent
- T: Temperature
- t: time

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The strength of this mathematical model was confirmed by the coefficients of determination R^2 = 99.2 %, which was statistically significant at $p < 0.0001$. Moreover, figure 5B shows the variation of the residual values and the normal probability that was used to confirmed that the model corresponds to the normal probability curve. According to this figure, it is possible to affirm that the model is adequate, because in the opposite case, the points would have been scattered in the graphic space and not distributed on the line of normal probability.

Overall, these results showed that ethanol can be added to anthocyanin extraction solvent in order to improve the yield of extraction. Ethanol is also the natural solvent for anthocyanins during winemaking process (Pekic *et al.*, 1998; Pirniyazov *et al.*, 2003) and is a reliable solvent for the food industry. The fact that ethanol concentration, in the extraction solvent, has an important influence on anthocyanin extraction has also been observed by Patil *et al.* (2009).

CONCLUSIONS

Only five anthocyanins were identified and quantified in Grenache noir leaves by HPLC-UV, which is a drastic difference compared to grape berries and wine in which fifteen different anthocyanins are generally identified. Among these five anthocyanins, cyanidin-3-*O*-glucoside and peonidin-3-*O*-glucoside were the main anthocyanins since they accounted for 44 and 37 % of all the anthocyanins, respectively, while delphinidin-3-*O*-glucoside, petunidin-3-*O*-glucoside and malvidin-3-*O*-glucoside accounted for only 6, 5 and 8 %, respectively. This was also a drastic difference compared to grape berries and wine in which malvidin-3-*O*-glucosides is the main anthocyanin.

Moreover, the best anthocyanin extraction condition from Grenache noir leaves using SO_2 in the aqueous extraction solvent was estimated by a multi-factorial design experiment, which revealed that the highest anthocyanin extraction yield would be obtained for an extraction time ranging between 5.77 and 6 h, a temperature ranging between 20 and 23.7 °C and a concentration of 500 ppm of SO_2 in the aqueous extraction solvent. Similarly, the highest anthocyanin extraction yield using an hydro-alcoholic extraction solvent was obtained for an extraction time ranging between 3 and 4.37 heures, a temperature set at 20 °C and an ethanol concentration in the extraction solvent ranging between 32 and 40 %.

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Table 3 - Values of the total anthocyanin concentration ([TAC]) for each experimental test and the weighting coefficients (Wc) for each factor as well as between-factor interactions

Factor	1	2	3	Effect 1*2	Effect 1*3	Effect 2*3	Effect 1*2*3	[TAC]
Test	Time	Temp	[EtOH]					(mg.g^{-1} dw)
1	-	-	-	+	+	+	-	5.81
2	+	-	-	-	-	+	+	5.41
3	-	+	-	-	+	-	+	5.05
4	+	+	-	+	-	-	-	4.80
5	-	-	+	+	-	-	+	6.12
6	+	-	+	-	+	-	-	5.86
7	-	+	+	-	-	+	-	5.50
8	+	+	+	+	+	+	+	5.24
Wc	-0.290	-0.654	0.408	0.036	0.034	0.030	-0.042	/

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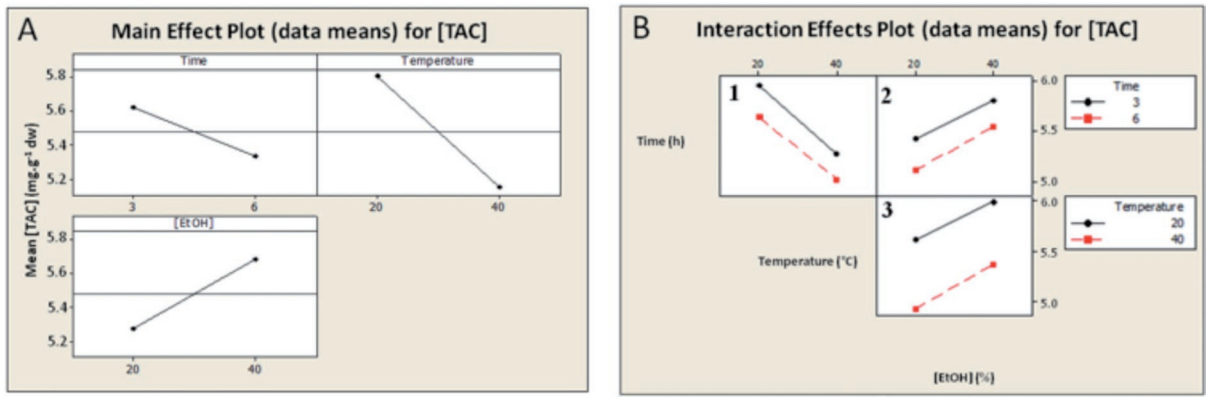


Figure 4 - A : Effects of the main factors on the total anthocyanin concentration ([TAC]) response; B: Interactions between factors as a function of TAC, with (1) Time/Temperature Interaction, (2) Time/[EtOH] Interaction; and (3) Temperature/[EtOH] Interaction.

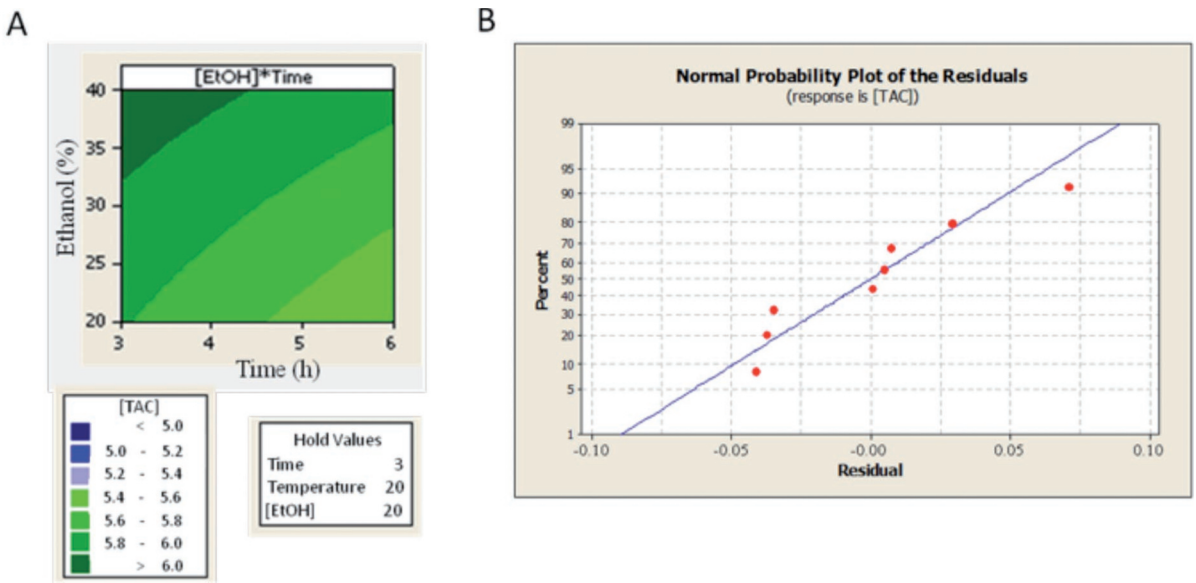


Figure 5 - A: Contour curve for total anthocyanin concentration ([TAC]) based on the [EtOH] and the time at a constant temperature (20°C); B: Normal probability plot of the mathematical model.

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