ANTHOCYANINS COMPOSITION AND EXTRACTION FROM GRENACHE NOIR (*VITIS VINIFERA* L.) VINE LEAF USING AN EXPERIMENTAL DESIGN II- BY ETHANOL OR SULFUR DIOXIDE IN ACIDIFIED WATER

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Abstract

Aim : Anthocyanins are water soluble pigments located in grape skin as well as in vine leaves. The aim of this work was to identify, and determine the anthocyanin composition in Grenache noir (*Vitis vinifera*) leaves as well as to estimate the optimum conditions leading to the highest anthocyanin extraction yield.

Methods and results : Five anthocyanins were identified and quantified in Grenache noir leaves by HPLC-DAD-MS. Among these anthocyanins, cyanidin-3-O-glucoside and peonidin-3-O-glucoside were the main anthocyanins, representing 43 and 38 %, respectively, while delphinidin-3-O-glucoside, petunidin-3-O-glucoside and malvidin-3-O-glucoside accounted for only 6, 5 and 8 %, respectively. To estimate the highest anthocyanin extraction yield, a multi-factorial design experiment was used to build a mathematical model. The extraction conditions tested were time, temperature and solvent composition (i. e., ethanol or SO_2 level in acidified water).

Conclusion : Only the five mono-glucoside anthocyanins usually detected in grapes have been detected, identified and quantified in Grenache noir leaves. The optimum extraction conditions of these anthocyanins were obtained for acidified hydro-alcoholic solution (i. e., pH 2, 3 h, temperature ranging between 36.5 and 40 °C and an ethanol concentration ranging between 36.8 and 40 %) and for acidified water with SO_2 (i. e., pH 2, between 5.8 and 6 h, a temperature ranging between 38.6 and 40 °C and a concentration of 500 ppm of SO_2).

Significance and impact of the study : Five mono-glucoside anthocyanins were identified and quantified in Grenache noir leaves. The optimum extraction conditions for of these anthocyanins were estimated in acidified hydro-alcoholic solution and in acidified water with SO_2. These extraction procedures will allow further investigation of the potential use of these anthocyanins as natural pigment for 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 hydrosolubles localisées dans la pellicule des raisins ainsi que dans les feuilles de vigne. Le but de ce travail a été d'identifier, de quantifier et de déterminer la composition des anthocyanes présentes dans les feuilles de Grenache noir (*Vitis vinifera*) ainsi que de déterminer les conditions optimum conduisant au meilleur rendement d'extraction des ces anthocyanes.

Méthodes et résultats : Cinq anthocyanes ont été identifiées et quantifiées dans des feuilles de Grenache noir par HPLC-DAD-MS. Parmi ces anthocyanes, la cyanidine-3-O-glucoside et la péonidine-3-O-glucoside étaient les anthocyanes majoritaires représentant respectivement 43 et 38 %, 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. Un plan d'expérience multifactoriel a été utilisé pour construire un modèle mathématique permettant d'estimer le meilleur rendement d'extraction des anthocyanes. Les paramètres étudiés ont été le temps, la température et la composition du solvant (SO_2 ou éthanol concentration dans eau acidifiée).

Conclusion : Uniquement les cinq anthocyanes mono-glucosides généralement identifées dans les raisins ont été détectées et quantifiées dans des feuilles de Grenache noir. Les conditions d'extraction optimum de ces anthocyanes ont été déterminées en solution hydro-alcoolique (pH2, 3 h, température entre 36.5 et 40 °C et concentration en éthanol entre 36.8 et 40 %) ainsi qu'en solution aqueuse contenant du SO_2 (pH 2, entre 5.8 et 6 h, température entre 38.6 et 40 °C et concentration de SO_2 de 500 ppm).

Signification et impact de l'étude : Cinq anthocyanes mono-glucosides ont été détectées, identifiées et quantifiées dans des feuilles de Grenache noir 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 naturel alimentaire 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 widespread in the plant kingdom, especially in angiosperms and flowering plants (Mazza and Miniati, 1993 ; Bridle and Timberlake, 1997). These polyphenolic compounds belong to the flavonoid family ; they are responsible for the red and blue color of plants and are found in high concentration in wine (McDougall *et al.*, 2005). These pigments are well known as natural red colorants (Kırca *et al.*, 2007), which make them important for the food industry (McDougall *et al.*, 2005). Moreover, these natural colorants are of interest due to their wide range of colors (i. e., deep red to deep blue), their high solubility in aqueous media, and their health benefits (Teszlak *et al.*, 2005). The latter include, among others, their antioxidant capacity (Rivero-Pérez *et al.*, 2008 ; Corrales *et al.*, 2009 ; Kırca *et al.*, 2007 ; Teissedre *et al.*, 1996), their vitamin P activity (Jonadet *et al.*, 1983), their protective activity against coronary heart disease (Gülçin *et al.*, 2005), their veinotonic effect and their anti-edematous action (Kiesewetter, 2002 ; Schaefer *et al.*, 2003).

Anthocyanins have been extracted from various plant sources and by-products, particularly from grape skins and pomace, to produce pigment for the food, cosmetic and medical industry. However, only few studies have focused on vine leaf as natural source of anthocyanins. These studies revealed that only the five mono-glucoside anthocyanins were observed in vine leaves of Carignan (Ezzili *et al.*, 1999), Merlot and Cabernet sauvignon (Darné and Glories, 1988) at leaf fall (Ezzili, 2001). Such anthocyanin composition is drastically different from the anthocyanin composition usually found in grapes, wines and pomaces. In grape berries and wine from *Vitis vinifera* L. varieties, there are fifteen different anthocyanins divided into three groups depending on the glucosidic group at position 3 (i. e., mono-glucoside, acetyl-glucoside and *p*-coumaroyl-glucoside), with 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 ; Lorrain *et al.*, 2012 ; González-Centeno *et al.*, 2012).

Despite the great potential of anthocyanins as natural pigment for the food, 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 these natural pigments. 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 properly studied. Therefore, the aim of this work was to evaluate the influence of temperature, time and solvent composition (i. e., ethanol or SO_2 level in acidified water) on the anthocyanin extraction yield from Grenache noir leaves. The experimental factorial design method was used to design this study, since 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 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 accuracy.

MATERIALS AND METHODS

1. General

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. Grenache noir grafted on Richter 99, which resulted from a massal selection Tunisian obtained for 35 years. This vine was planted in the Bir Bou Ragba area, Nabeul in Tunisia at latitude 10° 25' and longitude 36° 37W, 20 m above sea level. In the vineyard, vine spacing

was 2 m, while row spacing was 3 m. Fertilization was performed using commercial standards. The nitrogen contribution was carried out annually at a rate of 100 units/hectare in the form of ammonitrate. Phosphorus was brought at a rate of 300 kg of superphosphate/hectare before plantation. Similarly, potassium was brought at a rate of 300 kg/hectare before plantation in basic manure and then was annually brought at a rate of 100 kg/hectare. The vine leaves opposite the first cluster were collected on December 8, 2009.

After collection, the leaves were dried at 20 °C in a dark, ventilated room and then crushed with a Forplex crusher. The obtained powder was stored in the dark at 20 °C in black plastic bags until needed.

3. Extraction process

0.5 g of vine leaf powder was extracted in the dark by 50 mL of the extraction solvent as specified in Table I. The extraction solvents were acidified to pH 2 with hydrochloric acid. The factors considered were the extraction time (h), the extraction temperature (°C) and the solvent concentration. Each set of experiment was performed in triplicate. Prior to anthocyanin identification by HPLC-DAD-MS and quantification by spectrophotometry and HPLC-DAD, all the extraction mixtures were filtered through a 0.45-µm filter membrane.

4. Determination of the anthocyanin concentration

The determination of the total anthocyanin concentration (TAC) was performed by spectrophotometry using the differential pH 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 the sample in 2.4 mL of buffer solution). Such one-fifth dilution preserves the effect of buffer and stays within the range of reliable absorbance readings (DO < 1.5). The absorbances were measured at 520 nm and 700 nm 20 min after dilution in order to insure complete equilibrium between all the anthocyanin forms. TAC in each sample was calculated using the formula below and expressed in mg of anthocyanins per g of leaf powder dry weight (dw).

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

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
- 1: optical way (i. e., 1 cm)
- ε: 20653 $l.mol^{-1}.cm^{-1}$ the molar extinction coefficient of cyanidin-3-*O*-glucoside.

5. Determination of the anthocyanin composition in the leaves by HPLC-DAD-MS

0.5 g of leaf powder was 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 obtained 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-DAD to estimate the anthocyanin composition of the leaf extract. The anthocyanins were quantified by external standard calibration using cyanidin-3-*O*-glucoside as standard.

These HPLC-DAD 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) at a flow rate of 1 $mL.min^{-1}$ with a detection set at 520 nm. The anthocyanins were identified and assigned by comparison of their retention time, UV spectra and mass spectra with authentic standards using the same mass spectrometer system and methods as previously described (Kelebek *et al.*, 2010).

6. Experimental design

A complete factorial design was applied to identify the optimal conditions of anthocyanin extraction from vine leaves. The effects of three independent variables (i. e., solvent composition, extraction temperature and extraction time) on the anthocyanin concentration were investigated. The aqueous extraction solvents used were composed of different ethanol concentration (i. e., 20 % or 40 %) or different SO_2 content (i. e., 500 ppm or 2000 ppm). For each set of extraction, the extraction temperature tested was 20 °C or 40 °C and the extraction time was 3 h or 6 h. The experimental matrix was determined using MINITAB 14.0 software.

7. Statistical analysis

The data were submitted to one-way of variance analysis to test the influence of temperature, time and solvent composition on the anthocyanin extraction yield from Grenache noir leaves. Further, Duncan's multiple range tests were used to compare the mean values. All of the statistical analyses were performed with SPSS statistics software version 19.0 (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

1. Composition of anthocyanins extracted from Grenache noir leaves

The extracts obtained from Grenache noir vine leaves were analyzed using HPLC-DAD-MS and only five monomeric anthocyanins were identified based on their retention time at 520 nm and their mass fragmentation pattern. According to Kelebek *et al.* (2010), these anthocyanins were assigned to delphinidin-3-*O*-glucosides, cyanidin-3-*O*-glucosides, petunidin-3-*O*-glucosides, peonidin-3-*O*-glucosides and malvidin-3-*O*-glucosides. The main anthocyanins were cyanidin-3-*O*-glucosides and peonidin-3-*O*-glucosides since they represented 43 and 38 % of all the anthocyanins, respectively, while delphinidin-3-*O*-glucosides, petunidin-3-*O*-glucosides and malvidin-3-*O*-glucosides accounted for only 6, 5 and 8 %, respectively. Such anthocyanin composition has been previously observed and reported in Carignan (Ezzili *et al.*, 1999), Merlot and Cabernet-Sauvignon (Darné and Glories, 1988) leaves at leaf fall. However, it 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; Lorrain *et al.*, 2012; González-Centeno *et al.*, 2012). Furthermore, it appears that extraction condition such as time, temperature and composition of the acidified (i. e., pH 2) extraction solvent (i. e., SO_2 or ethanol) did not have a significant effect on the extracted anthocyanin compositions since the ratios between each anthocyanin remained similar, as previously observed (Nabli *et al.*, 2012).

2. Optimizing conditions for anthocyanin extraction using sulfur dioxide in acidified water

To optimize parameters of anthocyanin extraction from Grenache noir leaves, a complete factorial design of experiments was established. TAC was estimated for each experiment and reported in Table I: it was highest (i. e., 7.42 $mg.g^{-1}$ dw) with condition 4 (i. e., 6 h, 40 °C and 500 ppm of SO_2) and lowest (i. e., 6.33 $mg.g^{-1}$ dw) with condition 5 (i. e., 3 h, 20 °C and 2000 ppm of SO_2). Moreover, it was observed that the concentration of SO_2 had a significant negative impact on anthocyanin extraction since the conditions 5 to 8, with the highest SO_2 level, exhibited the lowest TAC.

The specific impact of each factor (i. e., time, temperature, SO_2 concentration) on TAC was estimated by comparing the curves of the average response of each studied factor (Figure 1A-C). The main effects of the three studied factors taken separately reveal that both time and temperature have a significant positive effect on TAC. In contrast, the increase of SO_2 concentration has a negative effect on TAC, as shown by the dramatic decrease compared to the other two curves. Indeed, TAC decreased by about 10 % (i. e., from 7.16 $mg.g^{-1}$ dw to 6.53 $mg.g^{-1}$ dw) when the concentration of SO_2 increased from 500 ppm to 2000 ppm in the extraction solvent (Figure 1C). The reaction between SO_2 and anthocyanins leading to the formation of colorless compounds might be the cause of this strong negative impact on the anthocyanin extraction yield, even if it has been previously observed that SO_2 increases anthocyanin extraction yield by improving the

Table I - Total anthocyanin concentration ([TAC]) for each experimental test

Condition	Time (h)	Temp (°C)	[SO_2] (ppm)	[TAC] ($mg.g^{-1}$ dw)
1	3	20	500	$6.95 \pm 0.03^{c/d}$
2	6	20	500	$7.18 \pm 0.06^{a/e}$
3	3	40	500	$7.11 \pm 0.05^{c/a/e}$
4	6	40	500	7.42 ± 0.10^{e}
5	3	20	2000	6.33 ± 0.27^{a}
6	6	20	2000	$6.50 \pm 0.09^{a/b}$
7	3	40	2000	$6.77 \pm 0.11^{b/c}$
8	6	40	2000	$6.53 \pm 0.02^{a/b}$

[a/b]Results are the means of three repetitions ± standard deviation.

a, b, c, d and e: different superscripts indicate that means differ ($p < 0.05$).

dissemination of anthocyanins through cell walls (Gao and Mazza, 1996).

The interaction between each factor was use to estimate the best extraction condition by looking at the response curve of opposite factors by pairs (Figure 1D-F). The results show that an extraction time of 3 h has a positive effect on the anthocyanin extraction yield when coupled with the temperature (Figure 1D) and a negative effect when coupled with an increase of SO_2 concentration (Figure 1E). Similar effects were observed with the extraction time of 6 h. Regarding the temperature, a positive effect on TAC was noticed when coupled with the extended extraction time (i. e., 6 h) and with the lowest SO_2 concentration (i. e., 500 ppm) (Figure 1 D-F). Moreover, in all the studied conditions (i. e., time and temperature), an increase of the SO_2 concentration from 500 ppm to 2000 ppm led to a decrease of TAC.

Finally, it was possible to determine the optimum conditions for anthocyanin extraction by plotting the 2D contour curve on the studied intervals for each factor. So, TAC would be highest (above 7.42 $mg.g^{-1}$ dw) for an extraction time ranging between 5.8 and 6 h, a temperature set between 38.63 and 40 °C and 500 ppm of SO_2 in the acidified (i. e., pH 2) extraction solvent (Figure 2A). Moreover, it was possible to obtain the predicated polynomial model below fitted in the three studied factors (i. e., time, temperature, SO_2) for the anthocyanin extraction yield.

$$[TAC] = 6.88 + 0.0378\,t + 0.0109\,T - 0.000422\,[SO_2]$$

($R^2 = 91$ %)

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

he strength of the mathematical model was confirmed by the coefficient of determination (R^2), which was 91 %. It was statistically significant at a level of $p = 0.015$. Moreover, according to Figure 2B, which shows the variation of the residual values and the normal probability that was used to confirm that the model corresponds to the normal probability curve, it is possible to affirm that the model is adequate ; in the opposite case, the points would be scattered in the graphic space rather than show a normal probability distribution.

Thus, according to these results, it was possible to conclude that SO_2 in acidified (i. e., pH 2) water can be added to anthocyanin extraction solvent in order to improve the yield of extraction, but in low concentration (i. e., 500 ppm). This conclusion is in agreement with previously reported data (Ayed *et al.* 1999 ; Nabli *et al.*, 2012), showing that SO_2 can enhance the extraction of anthocyanins by increasing their solubility (Cacace and Mazza, 2002).

3. Determination of the optimum anthocyanin extraction conditions using ethanol in acidified water

Following a similar strategy as above, the influence of ethanol level together with time and temperature on

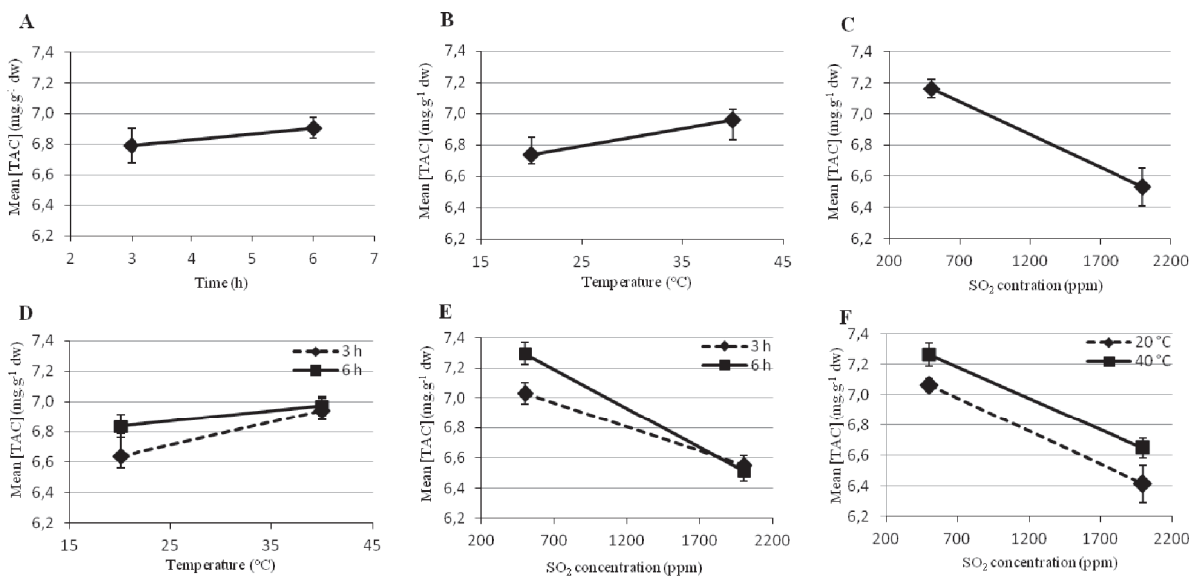

Figure 1 - Impact of the studied factors on the total anthocyanin concentration ([TAC]). A : Time, B : Temperature, C : SO_2 concentration, D : Time and temperature together, E : Time and SO_2 concentration together, F : Temperature and SO_2 concentration together.

TAC yield from Grenache noir leaves was then evaluated. Table II shows the TAC values obtained for each experimental test. The extraction condition 7 (i. e., 3 h, 40 °C, 40 % of ethanol) resulted in the highest amount of extracted anthocyanins (i. e., 5.69 $mg.g^{-1}$ dw), whereas the lowest amount (i. e., 4.78 $mg.g^{-1}$ dw) was observed with the extraction condition 1 (i. e., 3 h, 20 °C and 20 % of ethanol).

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 factors (Figure 3). Regarding the main effects of the three factors taken separately, it was noted that both temperature and ethanol concentration in acidified (i. e., pH 2) water have a positive effect on the anthocyanin extraction yield (Figure 3B-C), while extraction time has a negative effect (Figure 3A). As previously observed, the negative impact of the extended extraction time most likely resulted from the thermal degradation of the extracted anthocyanins, since longer extraction increases the risk of anthocyanin degradation.

By looking at the response curve of opposite factors compared in pairs, it was possible to evaluate the interactions between each factor. As shown in Figure 3D, an extraction time of 3 h has a positive effect on TAC yield when coupled with the temperature (Figure 3D) and when coupled with an increase of ethanol concentration (Figure 3E). Moreover, concerning the increased extraction temperature, it has been noted a positive effect on TAC when coupled with the shortest extraction time (Figure 3D) and with the highest ethanol concentration (Figure 3F). The positive influence of temperature on the amount of extracted anthocyanins might be due to the reduction in viscosity. Indeed, an increase in temperature

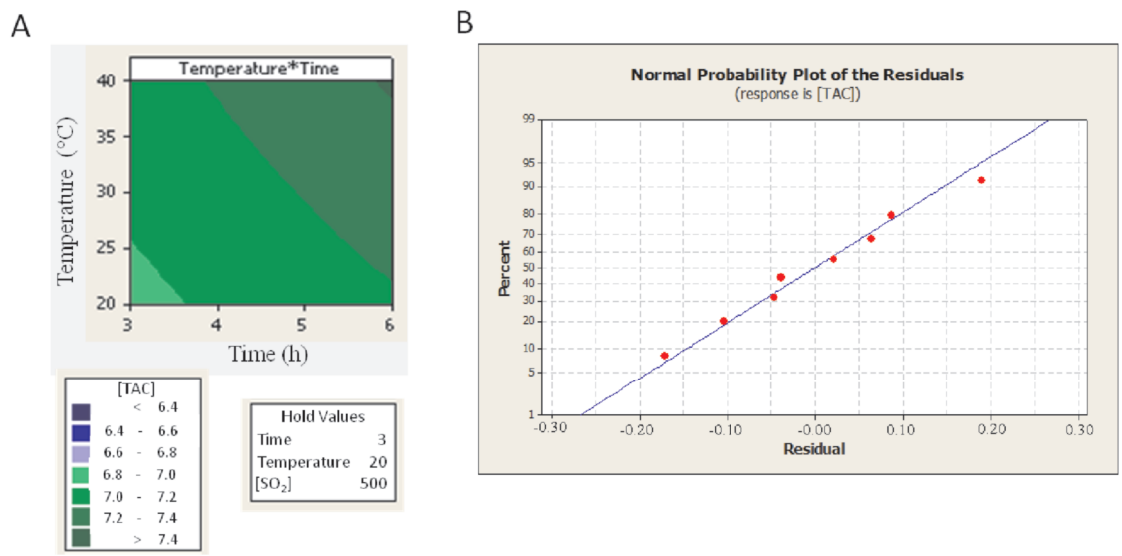

Figure 2 - A : Curve contour for total anthocyanin concentration ([TAC]) based on temperature and time at a constant $[SO_2]$ (500 ppm). B : Normal probability plot of the mathematical model.

Table 2 - Total anthocyanin concentration ([TAC]) for each experimental test.

Condition	Time (h)	Temp (°C)	[EtOH] (%)	[TAC] ($mg.g^{-1}$ dw)
1	3	20	20	4.78 ± 0.07^{a}
2	6	20	20	$4.86 \pm 0.13^{a/b}$
3	3	40	20	5.17 ± 0.04^{b}
4	6	40	20	$4.88 \pm 0.04^{a/b}$
5	3	20	40	5.20 ± 0.16^{b}
6	6	20	40	5.15 ± 0.15^{b}
7	3	40	40	5.69 ± 0.06^{c}
8	6	40	40	5.60 ± 0.13^{c}

Results are the means of three repetitions ± standard deviation.
a, b and c : different superscripts indicate that means differ ($p < 0.05$).

generally involves a reduction in viscosity, which thus significantly increases the diffusion of the molecules in solvent (Corrales *et al.*, 2009). The ethanol concentration in the acidified (i. e., pH 2) extraction solvent has an important influence on anthocyanin extraction; this condition has been extensively used in technological processes to extract anthocyanins from natural sources (i. e., fresh berries, grape pomace). The acidification of the anthocyanin extraction solvent has two main effects: first, the weakening of the membrane structures of the cells in which the anthocyanin are store in the vacuole and second, the transformation of the extracted anthocyanins to their flavylium form, which is their most stable form (Ben Amor, 2008).

Again, it was possible to estimate the optimum conditions for anthocyanin extraction by plotting the 2D contour curve on the studied intervals for each factor. Thus, Figure 4A shows that TAC would be highest (i. e., above 5.6 $mg.g^{-1}$ dw) for an extraction time of 3 h, a temperature ranging between 36.5 and

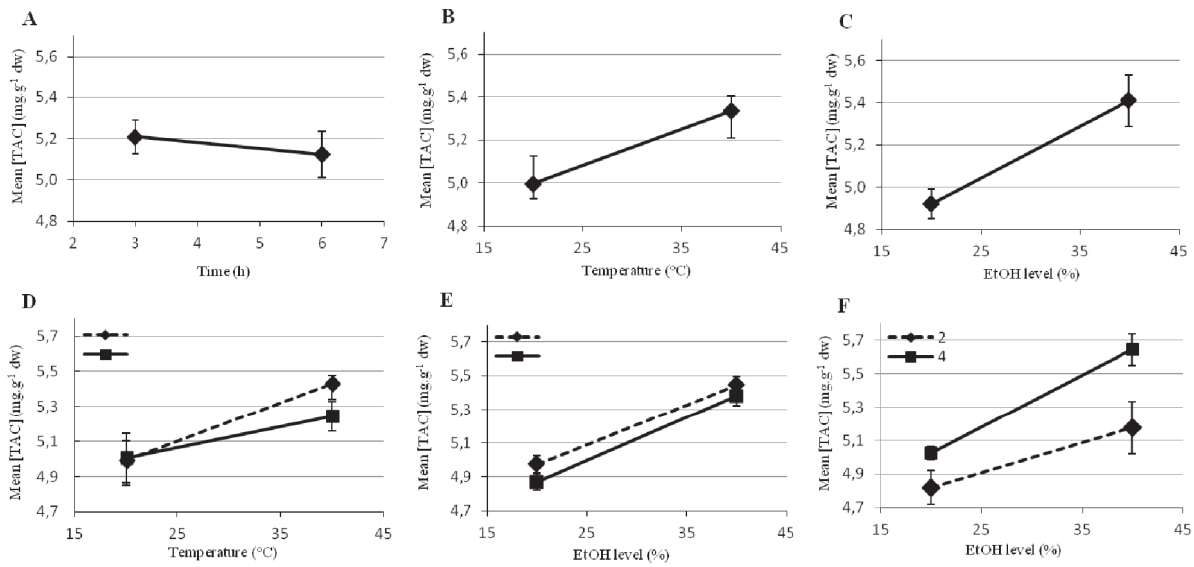

Figure 3 - Impact of the studied factors on the total anthocyanin concentration ([TAC]).
A: Time, B: Temperature, C: Ethanol level, D: Time and temperature together, E: Time and ethanol concentration together, F: Temperature and ethanol concentration together.

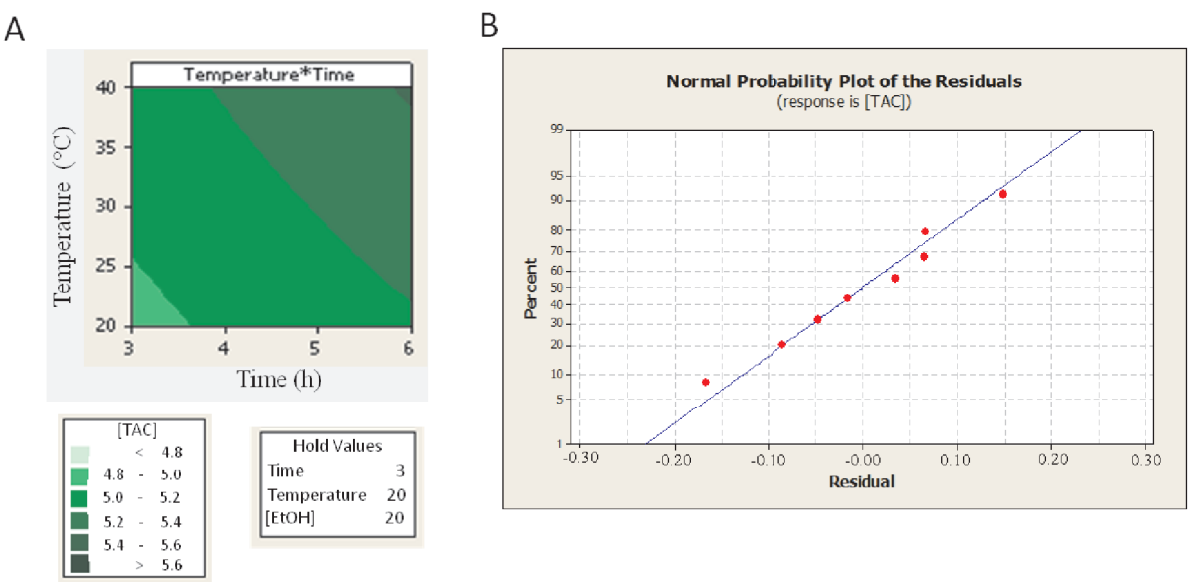

Figure 4 - A: Curve contour for total anthocyanin concentration ([TAC]) based on [EtOH] and temperature at a constant time (3 h). B: Normal probability plot of the mathematical model.

40 °C and an ethanol concentration in the extraction solvent ranging between 36.8 and 40 %. Moreover, 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.

$$[TAC] = 4.06 - 0.0283\ t + 0.0169\ T + 0.0244\ [EtOH] \quad (R^2 = 91.2\ \%)$$

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

The coefficient of determination R^2 (91.2 %) was statistically significant at a level of $p < 0.0001$. This result confirms the strength of the mathematical model. Moreover, the variation of the residual values and the normal probability are presented in Figure 4B. It was used to confirm that the model corresponds to the normal probability curve.

Overall, for the extraction with SO_2, an increase of time has a significant positive effect on TAC, while an increase of SO_2 concentration has a negative effect. So, a high TAC can be obtained with a low $[SO_2]_{ac}$ (i. e., 500 ppm) and a long extraction time (between 5.8 and 6 h). In contrast, for the extraction with EtOH, an increase of time has a negative effect on TAC, while an increase of EtOH in acidified water has a positive effect. So, a high ethanol concentration (between 36.8 and 40 %) and a short time (i. e., 3 h) can be used to obtain a high TAC. Concerning the extraction temperature, it was noted that it has a significant positive effect on TAC in both extractions with SO_2 and EtOH in acidified water. This conclusion is in agreement with reported data in few studies (Fan *et al.*, 2008; Cissé *et al.*, 2012), showing that temperature can enhance the extraction of anthocyanins by involving a reduction in viscosity, which thus significantly increases the diffusion of the molecules in solvent. However, a too high temperature coupled with a long time will result in a lower extraction yield due to the thermal degradation of the extracted anthocyanins.

Furthermore, it was noted that a high anthocyanin extraction yield was obtained by using a low $[SO_2]_{ac}$ (i. e., 500 ppm) or a high ethanol concentration (between 36.8 and 40 %). However, the concentrations of extracted anthocyanins using EtOH in extraction solvent were lower than with SO_2. Thus, it was possible to conclude that SO_2 in acidified (i. e., pH 2) water can be added to anthocyanin extraction solvent in order to improve the yield of extraction, but in low concentration (i. e., 500 ppm). This conclusion is in agreement with previously reported data (Ayed *et al.* 1999; Nabli *et al.*, 2012).

Moreover, the comparison of this set of data with the results previously obtained by Nabli *et al.* (2012) with non-acidified solvent shows that the anthocyanin extraction yield from Grenache noir leaves is higher with acidified solvent. Acidic solvents have been used in technological processes to extract anthocyanins from natural sources (i. e., fresh berries, grape pomace) (Ben Amor, 2008). The fact that acidified extraction solvent has an important influence on anthocyanin extraction has also been observed in berries by Byamukama *et al.* (2005) and Wu *et al.* (2011) and in vine leaves.

CONCLUSION

The impact of SO_2 concentration and ethanol level in acidified (i. e., pH 2) extraction solvent together with time and temperature on the anthocyanin extraction yield was investigated in Grenache noir leaves. Only five anthocyanins were identified and quantified by HPLC-DAD. Moreover, compared to the other anthocyanins, cyanidin-3-*O*-glucosides and peonidin-3-*O*-glucosides were the main anthocyanins since they accounted for 43 and 38 % of all the anthocyanins, respectively, while delphinidin-3-*O*-glucosides, petunidin-3-*O*-glucosides and malvidin-3-*O*-glucosides 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*-glucoside is the main anthocyanin.

Moreover, using a multi-factorial design experiment, the estimation of the best anthocyanin extraction conditions from Grenache noir leaves using SO_2 in the acidified (i. e., pH 2) aqueous extraction solvent were determined. It reveals that the highest anthocyanin extraction yield will be obtained for an extraction time ranging between 5.8 and 6 h, a temperature ranging between 38.63 and 40 °C, and 500 ppm of SO_2 in acidified (i. e., pH 2) water. Similarly, the highest anthocyanin extraction yield using an acidified (i. e., pH 2) hydro-alcoholic extraction solvent was obtained for an extraction time of 3 h, a temperature ranging between 36.5 and 40 °C and an ethanol concentration in the extraction solvent ranging between 36.8 and 40 %.

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