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Identification and quantification of stilbenes in some Tunisian red wines using UPLC-MS and HPLC-DAD

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Abstract

Seven Tunisian red wines mainly from the Mornag appellation were analyzed for resveratrol and analogues. The wines of each variety were evaporated, concentrated, and then subjected to fractionation and purification using XAD16 and DOWEX column chromatography. In addition to resveratrol, seven stilbenes were identified by UPLC-MS. The stilbenes derived were shown to be piceatannol, piceid, α-viniferin, ε-viniferin, hopeaphenol and isohopeaphenol. From the point of view of the presence of resveratrol derivatives, one wine, Sidi Zahia, was the richest qualitatively.

Keywords : stilbenes, red wines, HPLC-DAD, UPLC-MS

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Introduction

Numerous epidemiological studies since the 90s have shown that a very moderate wine consumption may be beneficial to health (Renaud *et al.*, 1999). The presence of polyphenols and particularly stilbenes in wine may explain these beneficial effects. Among stilbenes, resveratrol has demonstrated a number of promising biological activities, particularly in regards to disease prevention and anti-aging activities (Vang *et al.*, 2011 ; Catalgol *et al.*, 2012). Many studies have examined the resveratrol content in wine (Goldberg *et al.*, 1995; Mattivi and Nicolini, 1997). In contrast, few studies have investigated stilbene oligomers in wine. However, some studies indicate that wine contains many other stilbenes (Pawlus *et al.*, 2013 ; Moss *et al.*, 2013).

The aim of the present work was to develop a method to characterize stilbene content in Tunisian red wines. While many wines, especially French wines, have been studied for their contents in resveratrol, to our knowledge, no Tunisian wine has yet been explored in these regards. The presence of various stilbenes has been studied using UPLC-MS analysis on wine extracts. These compounds have been quantified by HPLC-DAD and different stilbenes have been identified, from the simplest monomers, such as resveratrol and piceid, to the more complex oligomers, such as e- and α-viniferin, hopeaphenol and isohopeaphenol. Concerning standards, if the simpler molecules may be obtained commercially, the complex stilbenes need to be purified from different plant sources. In this study, the standards were purified from vine parts and by-products from *Carex* species (Arraki *et al.*, 2013 ; Papastamoulis *et al.*, 2014).

In this study, seven Tunisian red wines mainly from the Mornag appellation were investigated. Seven stilbenes were identified in these wines : three monomers (resveratrol, piceid and piceatannol), one dimer (e-viniferin), one trimer (a-viniferin) and two tetramers (hopeaphenol and isohopeaphenol). Regarding the presence of resveratrol derivatives, we found that one of the wines, Sidi Zahia, was the richest qualitatively. These first results are very encouraging, and the study should be extended to a larger number of wines and appellations.

11. Reagents and standards

Methanol and acetonitrile were obtained from Scharlab® S.L. (Sentmenat, Spain), trifluoroacetic acid (TFA) from Sigma-Aldrich® and deuterated solvents from Euriso-Top. Water was purified using an Elga water purification system (Bucks, UK) with a resistivity of no less than 18 MΩ/cm.

2. Wine samples

Seven Tunisian red wines from the Mornag appellation (Clipea, Distinto, Mornag, Selian, Sidi Brahim, Sidi Rais and Sidi Zahia) were examined for their stilbene content by using UPLC-MS and HPLC-DAD methods. The wines were purchased from local stores and kept in the dark until analyzed.

3. Stilbene pre-purification

Red wine is a complex mixture of molecules. Among these, stilbenoids are in very low concentration and are often masked by other compounds, which sometimes makes it difficult to determine and quantify the stilbenes by techniques such as HPLC-DAD. Thus, in order to remove compounds such as sugars, anthocyanins or proanthocyanidins for example, we had to carry out a pre-purification step. The different wines were evaporated and purified on XAD16 and DOWEX columns (figure 1). These methods for the pre-purification of polyphenols have been developed and adapted from techniques already used in our laboratory and previously described concerning DOWEX columns (Decendit and Mérillon, 1996 ; Waffo-Téguo *et al.*, 1996) or XAD16 columns (Pawlus *et al.*, 2013). Each 750-mL bottle of wine was concentrated down to 650 ml *in vacuo* at 30 °C and diluted with 100 mL of water. The concentrated wine was poured over 500 g of Amberlite XAD-16 resin (Sigma-Aldrich), in an open column (5.1 cm id ´ 35 cm), and rinsed with 5 L of water to remove sugars, small organic acids and other non-polyphenolic compounds. The polyphenols were then eluted with 3 L of methanol and 1 L of acetone. The solvents were removed *in vacuo* and the samples were lyophilized. The concentrated XAD16 residue was then chromatographed over a cation-exchange resin column (DOWEX, Sigma-Aldrich), rinsed with distilled water (4 L), and eluted with 75 % (v/v) aqueous methanol (2 L) to yield a solid powder after lyophilization. In order to evaluate the accuracy of the purification method, we proceeded to verify the chromatographic steps with mini-columns (0.8 mm id ´ 5 cm) and two pure stilbenoid standards ((*E*)-resveratrol and (*E*)-piceid) ; the proportions between the amount of deposited stilbenes, the amount of resins, and the volumes of water and eluents for each step were conserved. For example, 10 µg of each stilbene were dissolved in 2 mL water and submitted to these two chromatographic steps in these conditions. The various steps were followed by HPLC as described below. We were not able to detect

stilbene in the rinsing waters of the columns, which means that there is apparently no loss of stilbenoids at this level. Elution with 75 % methanol at the last step with the DOWEX column allowed the recover 96.9 ± 0.9 % and 92.9 ± 1.6 % of piceid and resveratrol, respectively. These experiments were confirmed a second time. It should also be noted that, when the amount of stilbenes deposited on the columns was 10 times greater, we observed a loss of resveratrol in the rinsing waters during the step of the XAD16 column (approximately one third). Under these conditions, the limit of solubility of resveratrol in water is exceeded and we have a precipitate, which cannot be retained by the XAD-16 resin. These stilbene concentrations here (equivalent to 100 mg/L) are also much too high compared to those found in wine.

4. Analytical HPLC-DAD

Analyses were performed by analytical HPLC-DAD using a HPLC system model 1100 (Agilent technologies) coupled with a diode array detector (DAD). 20 µL of each sample was injected for each chromatographic analysis. Polyphenolic extracts were separated on a Bischoff® (Stuttgart, Germany) Prontosil® reverse-phase C_{18} column (5 µm packing, 4 mm id × 250 mm) protected with a guard column of the same material. The solvent system used was ultrapure water acidified with 0.1 % TFA (solvent A), and acetonitrile acidified with 0.1 % TFA (solvent B). The elution program at 1 mL/min was 10 % B (0-5 min), 10-60 % B (5-35 min), 60 % B (35-40 min) followed by a 5 min wash with 100 % B and a 5 min reequilibration step. The chromatograms were monitored at 290 and 306 nm and the spectra (200-600 nm) continuously recorded.

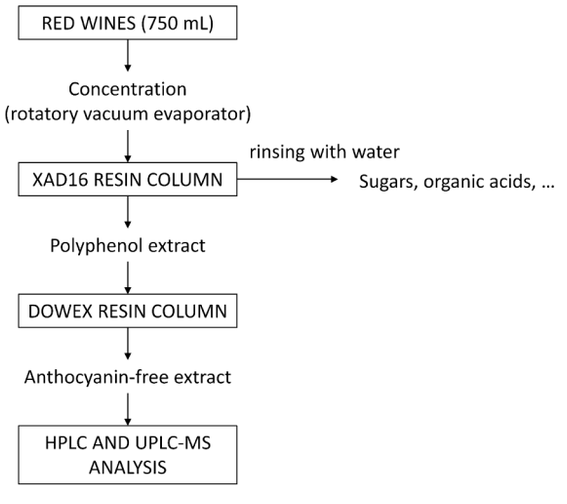

Figure 1. Flow diagram of primary method used for the different stages of pre-purification of wine stilbenoids.

5. UPLC-MS analysis

Monitoring of the collected Tunisian red wines was achieved by UPLC-MS. The UPLC analyses were carried out using an UPLC system model 1290 Infinity (Agilent Technologies) coupled to an Esquire 3000+ mass spectrometer (Bruker Daltonics GmbH) with electrospray ionization and ion trap analyzer. The column used was a C_{18} reversed-phase (100 × 2.1 mm, 1.8 µm, Agilent). Water 0.1 % formic acid (solvent A) and acetonitrile 0.1 % formic acid (solvent B) were used as mobile phases. 2.1 µL of each sample was injected and analyzed at 25 °C. The elution program at 0.21 mL/min was 10 % B (0-2 min), 10-60 % B (2-14 min), 60 % B (14-16 min) followed by a 2 min wash with 100 % B and a 5 min reequilibration step. The detection wavelengths were set at 290 and 306 nm. Electrospray ionization in positive and negative mode was used.

6. Stilbene quantification

Quantification of individual stilbenes was performed using standards/compounds isolated from Cyperaceae and Vitaceae. Quantification was performed by HPLC-DAD analysis on an Agilent® 1100 Series system by using the same HPLC-DAD conditions previously described. Extracts of red wines were dissolved at 1 mg/mL in methanol and 20 µl of these solutions were injected. The identity of each peak was verified in parallel with UPLC-MS for each wine sample. Stilbene concentrations were determined by reporting the measured integration area of each compound. Mean values of each extract were calculated from three replicates. For the calibrations, the same volume of injection was used. Seven solutions including blank were prepared for each pure isolated stilbenoid compound (0, 0.001, 0.005, 0.01, 0.02, 0.05 and 0.1 mg/mL in 50 % methanol). Each concentration was injected five times.

Results and discussion

1. Structure elucidation

In this study, stilbenes were identified by UPLC-MS/MS analysis. This combination provided an accurate method for the characterization of stilbenes (Stecher *et al.*, 2001). To illustrate the analysis of stilbenes in wine, the UPLC chromatogram of the Sidi Zahia red wine is presented in Figure 2. Stilbenes were tentatively identified by examining the mass spectra (MS and MS/MS). The presence of stilbene oligomers was monitored via extracted ion chromatograms in negative ion mode. Typical *m/z* values for these compounds were used, including

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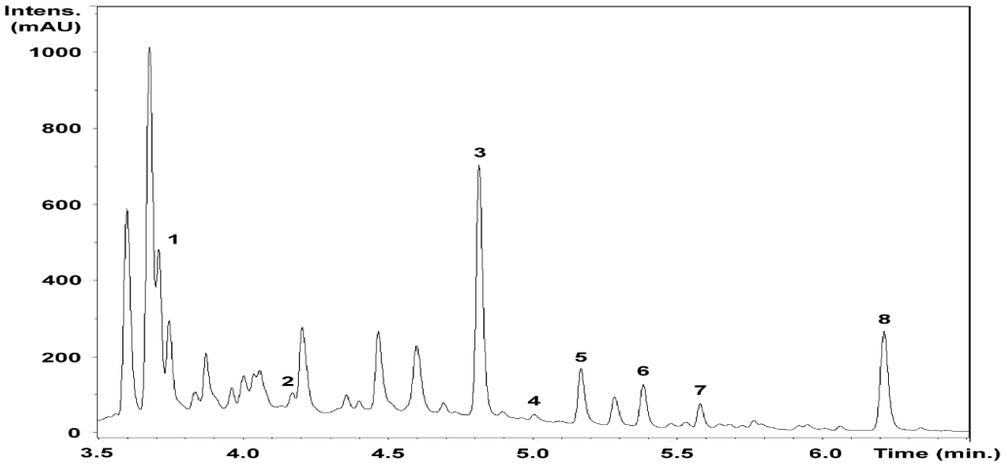

Figure 2. UPLC chromatogram recorded at 290 nm showing the stilbenes detected in the Sidi Zahia red wine. Peak numbering as in Table 1.

$[M-H]^-$ ions of *m/z* 227 or 242 (monomers), 453 (dimer), 677 (trimer), and 905 (tetramer). The corresponding values for these compounds were also evaluated in positive mode. To confirm the identification of the stilbenes, standards were used (Arraki *et al.*, 2013 ; Papastamoulis *et al.*, 2014).

Using this procedure, seven stilbenes (Figure 3) were identified in Tunisian red wines, including three monomers (resveratrol, piceid and piceatannol), one dimer (e-viniferin), one trimer (α-viniferin) and two tetramers (hopeaphenol and isohopeaphenol). Retention time (T_R) and molecular ions (positive and negative modes) are shown in Table 1.

Concerning the stilbene content in the Tunisian red wine, the Sidi Zahia red wine was qualitatively and quantitatively the richest one (Figure 3). The effective quantification of the stilbene content is in progress. Nevertheless, these studies show that among all studied wines, only Sidi Zahia contains the seven stilbenes (Table 2). Isohopeaphenol is present in all wines except Distinto. The Sidi Rais, Mornag, Sidi Brahim and Clipea wines contain four stilbenes and do not contain e-viniferin, a-viniferin and hopeaphenol.

2. Total stilbene contents

The average content of the seven stilbenes in the different red wines is shown in Table 3. The total concentrations of identified stilbenes were thus determined at 4.2 mg/L for Sidi Zahia, 2.5 mg/L for Sidi Rais, 3.9 mg/L for Mornag, 0.7 mg/L for Selian, 0.9 mg/L for Distinto, 1.5 mg/L for Sidi Brahim and 1.8 mg/L for Clipea.

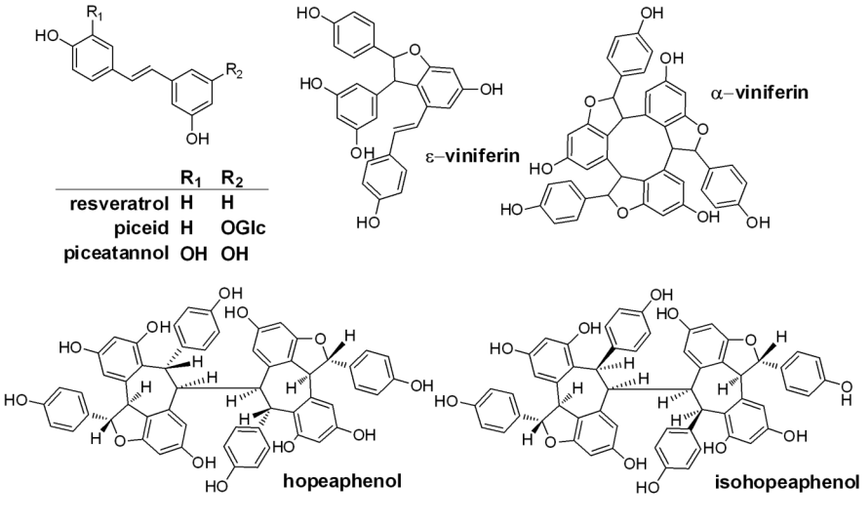

Figure 3. Chemical structures of various stilbenes identified in red wines.

Table 1. Identification of stilbene compounds in red wines using retention times and UPLC-MS data.

Peak	T_R (min)	$[M-H]^-$	$[M+H]^+$	Identification
1	3.7	389	391	(*E*)-piceid
2	4.2	242	244	piceatannol
3	4.8	227	229	(*E*)-resveratrol
4	5.0	677	679	a-viniferin
5	5.3	905	907	unknown
6	5.4	905	907	hopeaphenol
7	5.6	453	455	e-viniferin
8	6.2	905	907	isohopeaphenol

Table 2. Average detected stilbenes for Tunisian varietal wines (D : detected; ND : not detected).

Compound	Sidi Zahia	Sidi Rais	Mornag	Selian	Distinto	Sidi Brahim	Clipea
(*E*)-piceid	D	D	D	D	D	D	D
piceatannol	D	D	D	D	D	D	D
(*E*)-resveratrol	D	D	D	D	D	D	D
α-viniferin	D	ND	ND	ND	ND	ND	ND
hopeaphenol	D	ND	ND	D	ND	ND	ND
ε-viniferin	D	ND	ND	ND	ND	ND	ND
isohopeaphenol	D	D	D	D	ND	D	D

Table 3. Average content of stilbenes in red wines (mg/L).

Stilbenes	Sidi Zahia	Sidi Rais	Mornag	Selian	Distinto	Sidi Brahim	Clipea
(*E*)-piceid	0.30 ± 0.02	0.08 ± 0.01	0.20 ± 0.02	0.08 ± 0.01	0.10 ± 0.02	0.09 ± 0.01	0.09 ± 0.01
piceatannol	0.30 ± 0.03	0.10 ± 0.02	0.70 ± 0.04	0.20 ± 0.03	0.70 ± 0.04	0.20 ± 0.02	0.10 ± 0.02
(*E*)-resveratrol	0.30 ± 0.03	0.09 ± 0.01	0.10 ± 0.02	0.02 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.09 ± 0.01
a-viniferin	0.10 ± 0.01	-	-	-	-	-	-
hopeaphenol	0.20 ± 0.01	-	-	0.20 ± 0.02	-	-	-
e-viniferin	0.10 ± 0.01	-	-	-	-	-	-
isohopeaphenol	2.90 ± 0.20	2.20 ± 0.10	2.90 ± 0.30	0.20 ± 0.01	-	1.10 ± 0.10	1.50 ± 0.10

Conclusion

In this study, we were able to obtain seven Tunisian wines mainly from the Mornag appellation. We were able to determine in these wines the content of seven stilbenoids : (*E*)-resveratrol, (*E*)-piceid, piceatannol, e-viniferin, a-viniferin, hopeaphenol and isohopeaphenol. From the point of view of the presence of resveratrol derivatives, we found that one of the wines, Sidi Zahia, was the richest qualitatively. These first results are very encouraging. The study should now be extended to a larger number of wines and appellations.

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