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Influence of sample and mobile phase pH on HPLC-DAD-MS analysis of anthocyanins and other phenolic compounds in wine

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Abstract. In this study, the influence of pH of the wine sample (pH 1.2 and 3.3) and of the HPLC mobile phase (1 and 2 % formic acid in the solvent) on anthocyanin and phenolic acid analysis was explored. Wine samples acidified at pH 1.2 were analyzed immediately, after 30 min, 2h and 3.5h, in order to check if any degradation of phenolic compounds occurred during the storage. Maximum response of the flavylium cation of peonidin-3-glucoside (at 520 nm), statistically significant ($p < 0.001$), was observed in the sample with pH 1.2, and the hydrated forms were not detected under these conditions. The peak area of the flavylium cation was also slightly higher when elution was performed by 2 % formic acid in the HPLC solvent, but the differences were not significant ($p > 0.05$), meaning that both 1% and 2% formic acid in the mobile phase are suitable for anthocyanin analysis. Regarding phenolic acids, lower peak intensities, statistically significant ($p < 0.001$), were obtained in the negative ion mode with 2 % formic acid in the mobile phase, presumably due to the lower proportion of acids in the anionic form under more acidic conditions. Thus, 1% formic acid in the mobile phase and acidification of the sample at pH ≤ 1.2 were selected as the best compromise for simultaneous analysis of anthocyanins and phenolic acids. Acidification of the wine samples at pH 1.2 followed by a 30 min delay enabled total transformation of chalcone and carbinol forms into the corresponding flavylium cations, without causing degradation of the other wine phenolics. The optimized conditions were applied for MS identification of 69 compounds from different phenolic groups present in Vranec wine.

Introduction. The colour of anthocyanins is directly linked to pH of the medium [1,2,3]. In highly acidic media, flavylium cations are mainly present, losing their colour as the pH increases, as a result of their conversion to colourless carbinol pseudobases, which in turn are in equilibrium with the open ring chalcone yellow forms. In neutral and alkaline medium, the flavylium undergoes deprotonation yielding a purple quinoidal anhydrobase. The co-existence of different forms of anthocyanins in equilibrium leads to broadening of the elution peaks, poorer resolution, lower response in the visible range and less reproducible quantification of anthocyanins when performing the HPLC analysis and intricates the chromatogram at 360 nm resulting in difficult quantification of flavonols. To avoid this and maintain the anthocyanins in their flavylium form during analysis, low pH is required, which on the other hand, is not appropriate for detection of carboxylic acids as their carboxylate anions by mass spectrometry [4].

The main objective of this study was to develop an HPLC-DAD-MS method for separation and quantification of phenolic compounds in wine and especially to find a suitable compromise for simultaneous analysis of anthocyanins and phenolic acids. Stability of phenolic components was checked after storage of the wine samples and the optimized conditions were applied for HPLC-MS analysis of wine.

Materials and Methods. Wine was produced from Vranec grapes (vintage 2007). Extraction of skins from Alicante Bouché variety was performed with methanol/water/HCl (30:69.9:0.1, *v/v/v*), followed by solid-phase extraction on a cartridge with Toyopearl TSK gel HW-50 (F). Two acidified solutions of anthocyanin extract were prepared: one solution with pH 1.2, and the other one with pH 3.3. Wine samples were acidified with a solution of HCl (pH <0) so as to give solutions with final pH 1.2 and analyzed by HPLC immediately and after storage for 30 min, 2h and 3.5h at room temperature. HPLC-DAD-MS analyses were performed with Waters 2690 system equipped with DAD and ion trap MS. Separation of the components was performed on Atlantis dC18 column (250x2.1 mm, 5 μm). Mobile phase consisted of water/formic acid (99:1) and acetonitrile/water/formic acid (80:19:1) at a flow rate of 0.25 mL/min at 30 °C, using gradient elution profile. Chromatograms were recorded from 200 to 600 nm. ESI-MS data ranging from *m/z* 200 to 1200 were recorded in negative and positive ion modes.

Results and Discussion. Solutions at pH 1.2 and 3.3 of an anthocyanin extract were analysed by HPLC, using mobile phases containing 1 and 2 % (*v/v*) formic acid. The proportions of chalcone and flavylium forms of anthocyanins under the conditions tested were estimated from the peak areas of peonidin-3-glucoside and its chalcone, which did not show coelution with other components. Comparison of the chromatograms of skin extract solutions at pH 1.2 and 3.3, recorded at 360 nm (Fig. 1) showed the presence of the chalcone forms of malvidin-3-glucoside and peonidin-3-glucoside in the sample with pH 3.3, confirmed by their UV/Visible and their MS spectra ($[M+H]^+$ at *m/z* 511 and 481, respectively), but not at pH 1.2 ($p < 0.001$). Maximum response of flavylium cation of peonidin-3-glucoside (at 520 nm) was observed in the sample with pH 1.2. Its peak area was slightly higher with 2 % formic acid in the HPLC solvent, but analysis of variance showed that the differences were not significant ($p > 0.05$), meaning that 1 % and 2 % formic acid in the mobile phase are equally suitable for anthocyanin analysis.

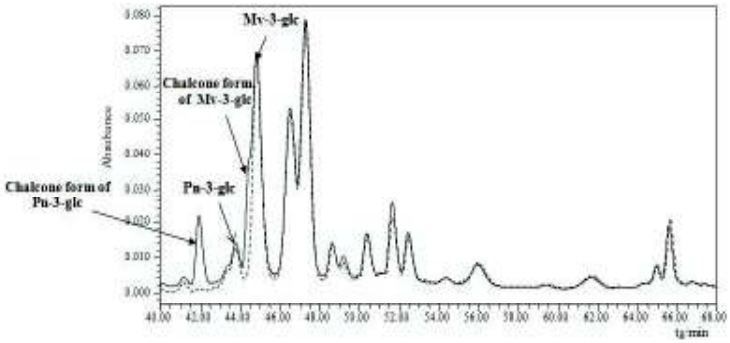

Figure 1. Chromatograms of the anthocyanin extract solutions at pH 1.2 (--) and 3.3 (—)

To check if degradations of phenolic compounds occurred during the storage of acidified samples, such as hydrolysis of flavonol glycosides, esterification of phenolic acids, hydrolysis or *trans* esterification of hydroxycinnamic esters and acid-catalysed cleavage of proanthocyanidins, a Vranec wine sample was adjusted to pH 1.2 and analyzed immediately, and after 30 min, 2h, and 3.5h of storage. The anthocyanin chalcone forms of malvidin-3-glucoside and peonidin-3-glucoside and their carbinol bases were absent in the wine sample acidified to pH 1.2 after 30 min storage of the wine sample. It was concluded that acidification of the wine samples at pH 1.2 followed by a 30 min delay enables total transformation of chalcone and carbinol forms into their flavylium cations, without causing degradation of the other wine phenolics. No further changes of the peak areas were observed with longer storage.

Lower peak intensities for phenolic acids were obtained in the negative ion mode with 2 % formic acid in the mobile phase ($p < 0.001$) presumably due to the lower proportion of acids in the anionic form under more acidic conditions [4]. From these data, 1 % formic acid in the mobile phase and acidification of the sample at pH ≤ 1.2 seems to be the best compromise for simultaneous analysis of anthocyanins and phenolic acids.

The optimized conditions enabled successful MS identification of 69 phenolic compounds from different groups in Vranec wine.

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