Validation of a routine HPLC method for added fumaric acid determination in wines

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Abstract. Nowadays, grape berries mature earlier due to climate change. Higher sugar contents are observed, whereas lower ones in organic acids, leading to increased pH values. Those biochemical changes have direct consequences on the balance and quality of wines made from such grapes. Chemical acidification, commonly used to compensate for the lack of acidity in musts or wines, includes tartaric, malic, lactic, and citric acids. Fumaric acid (FA), naturally present in the grape berries at low concentrations and already authorized in the member states of the OIV to inhibit malolactic fermentation in wines, seems to be a promising alternative to those acids to lower the pH. However, the evolution of FA levels added at bottling and its impact on wine quality during its conservation have not yet been studied. Therefore, the aim of the present study was to develop and validate a simple method using liquid chromatography coupled with a diode array detector, which can be used routinely for the determination of the added fumaric acid in wines. The proposed and validated method uses a ProntoSIL C-18 analytical column and an isocratic elution with water acidified at 0.1% formic acid. The run time, including column cleaning with acetonitrile and re-equilibration, was 40 minutes.

1 Introduction

Climate change has brought about important changes in the development of the vine (*Vitis vinifera L.*), thus resulting in several modifications on the level of grape berries composition. Notably, due to the increasing temperatures, grapes mature earlier, leading to higher contents in sugars and lower ones in organic acids [1, 2]. These biochemical changes in the berry can have direct consequences on the sensory balance and the quality of wines [3].

To compensate for the lack of acidity in musts or wines, winemakers commonly use chemical acidification, the process of artificially increasing the acidity of wines by adding organic acids [4]. The four acids authorized by the OIV are tartaric, malic, citric, and lactic acid. They can be added in musts and wines except for citric acid, which is only authorized in wines.

One of the possible alternatives to these existing acidification means could be the use of fumaric acid (FA) in musts and wines. FA is an organic acid naturally present in small quantities in certain fruits, including grape berries, found at concentrations between 0.07 and 10.69 mg/L [5-7]. Low FA quantities are also present in wines, less than 31 mg/L [6, 8] .It is already used for acidification in oenology in the United States (3 g/L maximum), in Canada and Chile, but not yet in the member countries of the OIV. Recently, it has been authorized in the member states of the OIV to inhibit malolactic fermentation in wines at concentrations between 300 and 600 mg/L, but not yet for acidification.

A great number of studies related to the determination of organic acids, including FA, in wines have been conducting during the past years. Various approaches are proposed, such as capillary electrophoresis [9], valve-switching ion chromatography [10] or direct infusion electrospray ionization mass spectrometry [11]. A recent work that presents particular interest is that of Fernández-Vázquez et al. [12]. Their study proposed an efficient enzymatic method, intended for the simultaneous determination of L-malic acid and fumaric acid in wines. Such a method can be useful for routine analysis of FA, but seems to lack precision, especially when compared to liquid chromatographic methods.

HPLC methods constitute the most common techniques used for organic acid analyses, as they are accurate and reliable. They used different modes such as ion exclusion HPLC [13, 14], LC-MS-MS [15] or reversed phase HPLC. There is currently a method for determining organic acids by reverse phase HPLC (RP-HPLC), including FA, published by the OIV (method OIV-MA-AS313-04, [16]). This method uses two C-8 columns placed in series with solvents adjusted to pH 2.1, guaranteeing optimal conditions for only 4 weeks of full-time use. Such a routine system can be costly in the long term.

In this context, the aim of this study was to develop and validate a simple method using liquid chromatography coupled with a diode array detector, which can be used routinely for the determination of the added FA in wines (white, rosé, and red).

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2 Material and methods

2.1 Chemicals

FA of analytical grade added to wines was purchased from Sigma Aldrich (Saint Luis, Missouri, United States). Ultrapure water (Milli-Q purification system, Millipore, France) was used for all solutions and chromatographic separation. The acetonitrile and formic acid used for chromatographic separation were purchased from Agilent Chemical (France).

2.2 Wines

The experimental assays for the development and the validation of the liquid chromatographic method were carried out using commercially available white, rosé and red wines (bag in box) free from FA.

2.3 Sample preparation

FA was added and dissolved in wine samples (white, rosé, red) at 1 g/L. The samples were first diluted 1/10 with water and then, successive dilutions in a wine: water (1:10) solution allowed to obtain a wide range of concentrations. Finally, the samples were filtered through 0.45 µm filters before injection in the HPLC.

2.4 HPLC-DAD analysis

FA determination in wine samples was carried out according to Flores et al. [15] in a Vanquish HPLC system consisting of a vacuum degasser, an autosampler, a sample thermostat and a binary (ThermoFischer Scientific, Waltham, MA, USA) equipped with a DAD (Thermo-Finnigan, ThermoScientific). A ProntoSIL C-18 analytical column of 250 mm × 3 mm and 3 µm particle size (Bischoff, Leonberg, Germany) was used. The solvents (A) and (B) were water and acetonitrile respectively, acidified with formic acid 0.1%. Solvent (B) was used to clean the column from phenolic compounds. A re-equilibration step followed, to reestablish the initial conditions before the next injection. The solvent gradient was: 0% B from 0 to 16 min, 100% B at 18 min for 6 min, 0% B at 26 min for 14 min. The injected sample volume was 10 µL and the flow rate was set at 0.4 mL/min.

2.5 HPLC method validation

The method was validated according to OIV-oeno 418-2013 [17] by studying linearity, limits of detection (LOD) and quantification (LOQ), intraday repeatability, inter-day precision, accuracy and recovery rate in white, rosé and red wines in which FA was added at known concentrations.

2.5.1 Linearity

Method linearity was studied by plotting the peak area for each FA concentration level versus the nominal concentration of each calibration standard. Good method linearity is established when $R^2 \geq 0.999$.

2.5.2 Limits of detection and quantification

The LOD and LOQ were estimated according to the signal to noise (S/N) method. More precisely, LOD was defined as the concentration where S/N = 3 and LOQ as the concentration where S/N = 10.

2.5.3 Repeatability and reproductibility

To evaluate the intraday repeatability, three replicates of three FA levels were injected for each wine on the same day and the RSD was calculated. Similarly, inter-day precision was determined by injecting two samples of different FA concentrations over five successive days. For the method to present good repeatability and reproducibility, RSD must be less or equal to 2% in both cases.

2.5.4 Accuracy

The accuracy of the method was evaluated by comparing the theoretical FA content in samples of known concentration, to the concentration that was calculated by applying the obtained linearity equations. The method was considered as accurate when the slope of the graph (calculated concentrations vs. theoretical concentrations) was equal to one, therefore signifying that the theoretical concentrations were equal to the calculated ones, and the y-intercept was equal to zero.

2.5.5 Recovery rate

In the case where FA concentrations in wine were too low according to the linearity range, the samples could be first purified on C18-SPE cartridges (Merck, France), to eliminate the main phenolic fraction susceptible to interfere with the quantification. A known volume of wine (V_i) was first evaporated to dryness under vacuum, re-solubilized in water and then deposed on a C18-SPE cartridge (Merck, France). The column was washed with water and the recovered aqueous fraction was evaporated to dryness under vacuum. A known volume of water was added (V_f) to obtain a FA concentration in the final extract (C_f) within the linearity range. Finally, the extracts were filtrated through 0.45 µm filters and injected in the HPLC to determine C_f. The initial FA concentration in wine (C_i) was obtained by multiplying C_f by the dilution factor $DF = V_f/V_i$. To estimate here the recovery rate, the theoretical C_i of FA in wines was 0.01 g/L and the DF was equal to one.

2.6 Statistical analysis

Linear regression analyses were performed with Excel Regression Analysis Tool.

3 Results and Discussion

3.1 HPLC method development

The developed methodology for the determination of FA in wine samples was based on Flores et al. [15]. The stationary phase used was a ProntoSIL C-18 column, which is suitable for the analysis of organic acids, as it is stable under 100% aqueous mobile phases and low pH ranges. Moreover, solvent acidification allows the best interaction with the non-polar stationary phase for an optimum resolution of acidic compounds. Thus, the best results related to method sensitivity and separation were achieved using an isocratic elution with water acidified at 0.1% (v/v) formic acid (pH = 2.3), at a flow rate of 0.4 mL/min and room temperature. A cleaning step using acidified acetonitrile (formic acid 0.1%) was added to the HPLC method to ensure the total elution of phenolic compounds. The injected sample volume was 10 µL and the run time, including column cleaning and re-equilibration, was 40 minutes. The mobile phase gradient program is described in paragraph 2.4. Under the current chromatographic conditions, FA eluted in all samples before 13 minutes (Fig. 1).

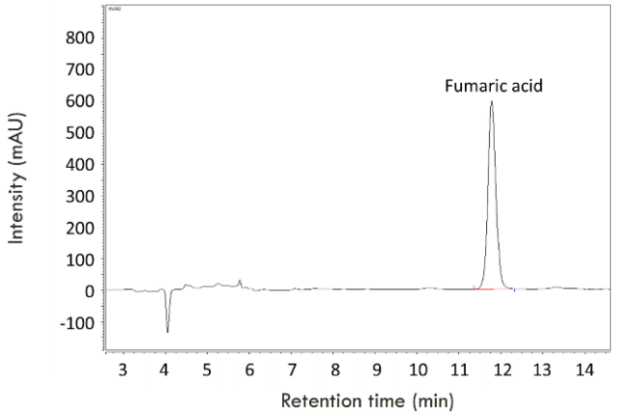

Figure 1. Example of chromatogram of fumaric acid in Rosé wine.

FA detection was performed with a DAD at 235 nm, as this wavelength provided a more stable signal at this wavelength than at the maximum absorbance wavelength of 205 nm (Fig. 2) and a smoother baseline in the chromatograms (Fig. 2).

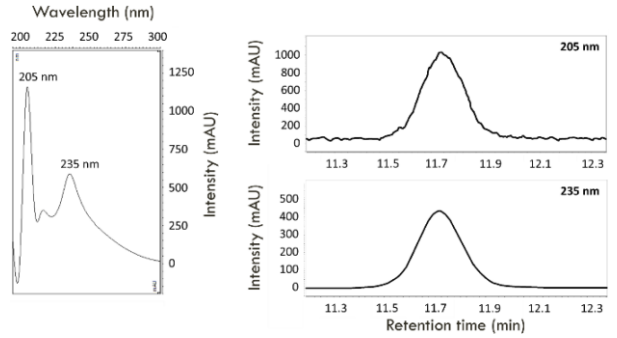

Figure 2. Fumaric acid spectrum and its chromatograms at 205 and 235 nm.

3.2 HPLC method validation

3.2.1 Linearity

The obtained curves presented a good correlation coefficient (R^2 > 0.999) for a concentration range from 0.3 mg/L to 75 mg/L for all matrixes examined, white, rosé and red wine as well as water (Table 2). The results, therefore, suggest that the different matrixes do not importantly affect FA quantification and that wine samples diluted at least 10-fold resemble to aqueous solutions containing FA.

Table 1. Linear equations and correlation coefficients (R^2) for fumaric acid in differet wine matrixes.

Matrix	***Equation***	***R^2***
White wine	y = 1764.6x	0.9994
Rosé wine	y = 1792.2x	0.9993
Red wine	y = 1745.6x	0.9993
Water	y = 1777.6x	0.9994

3.2.2 Limits of Detection and Quantification

In all the different matrixes, LOD for FA was found at 0.2 mg/L and LOQ at 0.3 mg/L.

3.2.3 Repeatability and reproducibility

Both intraday repeatability and inter-day precision presented an RSD ≤ 2% for all wine samples, thus ensuring good repeatability and reproducibility of the method (Table 2).

Table 2. Intraday repeatability and inter-day precision (%RSD) for different concentrations of fumaric acid.

Matrix	Intraday repeatability (% RSD)	Inter-day precision (% RSD)
White wine	0.122 (7.5 mg/L)	1.079 (50 mg/L)
	0.945 (10mg/L)	1.397 (75 mg/L)
	0.619 (50 mg/L)	
Rosé wine	1.421 (1 mg/L)	0.847 (5 mg/L)
	0.868 (5 mg/L)	0.911 (7.5 mg/L)
	1.033 (7.5 mg/L)	
Red wine	1.987 (5 mg/L)	0.905 (10 mg/L)
	1.205 (10 mg/L)	1.069 (75 mg/L)
	0.755 (75 mg/L)	

3.2.4 Accuracy

Figure 3 shows the plots of the calculated concentration of FA *vs*. its theoretical concentration for the different wine matrixes. Similar results were obtained for all wine matrixes, demonstrating once again that the medium does not significantly affect FA quantification.

Accuracy of the method was established as the slopes were almost equal to one for each plot and all the y-intercepts were equal to zero, according to the linear regression analysis that was performed.

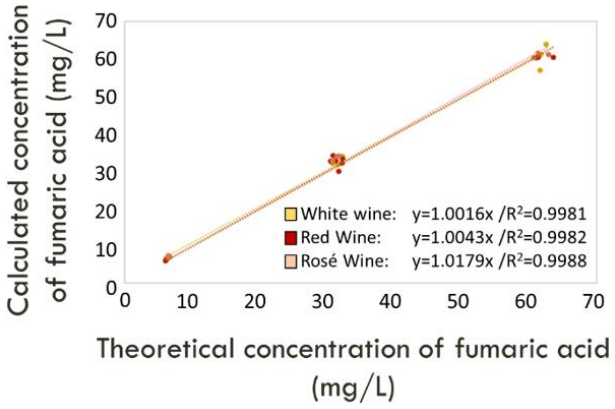

Figure 3. Calculated vs. theoretical fumaric acid concentration in white, red and rosé wines.

3.2.5 Recovery rate

The recovery rate in the studied wine samples were found between 106 and 118%, therefore within the acceptable range for analytical methods (Table 3).

Table 3. Recovery rate for fumaric acid in different matrixes.

Matrix	***Recovery rate (%)***
White wine	113.626
Rosé wine	117.593
Red wine	106.610

4 Conclusions

Overall, the proposed analytical methodology allows the quantification of a wide FA concentration range in different types of wine, with great efficacy. The HPLC method presents several advantages, such as minimum sample preparation, low organic solvent consumption, cost efficiency, high accuracy, and precision. It could therefore be used easily, in a routine way in wineries over an extended period, to measure FA in acidified wines.

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