

## New enzymatic method for estimating fumaric acid in wines

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### ABSTRACT

This work aims to develop a new enzymatic method for analysing fumaric acid in wines. Fumaric acid is a very effective lactic acid bacteria inhibitor that is widely used in the food industry as an additive (E297). Using it to inhibit malolactic fermentation has recently been authorized by the International Organisation of Vine and Wine (OIV). However, the official analytical method for fumaric acid involves the classical HPLC method for organic acids, which is overly complicated for use in wineries. This research proposes a very simple and efficient enzymatic method that allows the simultaneous determination of L-malic acid and fumaric acid. The method uses a commercial enzymatic kit for L-malic acid and adds a supplementary step in which the fumarase enzyme is added to transform fumaric acid into L-malic acid. The results obtained show that this method could be used in different media (synthetic solution, white wine, red wine and white grape juice). This article also proposes a modification of the enzymatic method to be applied when the L-malic acid concentration of the sample is very high

### KEYWORDS

fumaric acid, L-malic acid, enzymatic method

## INTRODUCTION

Fumaric acid (FA) is the common name of an organic acid named according to IUPAC nomenclature as (2*E*)-But-2-enedioic acid (CAS number 110-17-8). It is present in small quantities in a wide range of biological systems because it is an intermediary metabolite of the citric acid cycle (CAC). Following the normal flow of the CAC, FA is converted into L-malic acid (MA) through the action of the fumarase enzyme in the cytosol and mitochondria (Akiba *et al.*, 1984; Pines *et al.*, 1996). It presents as a white solid, with sharp acidity and low solubility in water (between 5 and 7 g/L at room temperature (Jamalzadeh *et al.*, 2012; Sebastian *et al.*, 2019; Straathof and van Gulik, 2012) but higher solubility in ethanol at 95 % (46 g/L at 25 °C). Its carboxylic acid groups are in *trans* (*E*) position and it is recognised as a safe product by various health organizations (EFSA, 2013; FDA, 2020).

FA has been widely used in industry since 1946 due to its acidifying properties in foods and juices (Straathof and van Gulik, 2012), sometimes substituting the use of citric or tartaric acid. The sensory threshold of FA is around 0.6 g/L in red wine and it seems that its presence may contribute to reducing wine pH, thus improving wine freshness and, in the case of red wines, colour intensity (Morata *et al.*, 2020).

To date, it has not been used in oenology because it has been very recently authorized by the OIV and it will presumably be authorized shortly by the European Union. However, it is widely used in the food industry (Additive E297) as an acidifier and most especially as a lactic acid bacteria inhibitor (European Union, 2008) up to dosage levels of 4 g/Kg or L. Indeed its inhibitory effect against certain microorganisms such as lactic acid bacteria (LAB) has been known since the 1970s (Pilone *et al.*, 1974). Its effectiveness depends on the microorganism species, dosage and other factors such as pH. A synergistic effect of fumaric acid on pH has been observed against *Oenococcus oeni*. At low pH 3.5, the inhibitory effect on LAB growth in wine is higher than it is at pH 4.1 (Pilone *et al.*, 1974). According to Morata *et al.* (2020), it has been recently reported a complete inhibition effect on *O. oeni* growth adding relatively low doses (0.3 to 0.6 g/L) at 3.3 pH.

Of the microorganisms present in wines, it seems that FA inhibits only lactic acid bacteria and not acetic acid bacteria or yeasts (Pérez-Díaz, 2011; Straathof and van Gulik, 2012).

In fact, *Saccharomyces cerevisiae* can metabolise FA, although its metabolic pathways have not been fully investigated. It is known that FA can be uptaken by passive diffusion and also by certain unidentified membrane transporters (Jamalzadeh *et al.*, 2012). *S. cerevisiae* cannot accumulate large amounts of FA in the cytosol. One reason for this may be that the cytosolic fumarase enzyme mainly catalyses the conversion of fumaric acid into L-malic acid but not vice versa, and uses it as a carbon source in anaerobic conditions (Pines *et al.*, 1996).

In view of the above, the possible use of FA as a specific inhibitor of LAB has recently been proposed by a number of delegations of the International Organisation of Vine and Wine (OIV). Recently, the resolution regarding the use of FA at a maximal dose of 0.6 g/L in wines to inhibit malolactic fermentation (Resolution OENO-TECHNO 15-581A) has been approved at the 19th OIV General Assembly that took place in Paris on July 12, 2021 (OIV, 2021).

It is therefore obvious that wineries will need an analytical method for estimating fumaric concentration in wines. According to the OIV, there are two different methodologies for analysing organic acids that can be considered for FA analysis: high-pressure liquid chromatography (HPLC) (OIV, 2009) and capillary electrophoresis (OIV, 2011). Both are effective for FA analysis but they cannot easily be applied by wineries because they need expensive equipment and specialised technicians. Other organic acids present in wine are usually analysed by wineries using commercial enzymatic kits that require only a spectrophotometer and the usual volumetric glassware. Specifically, these organic acids are L-malic acid, L-lactic acid, citric acid and succinic acid (Bergmeyer and Möllering, 1974) and even acetic acid (McCloskey, 1976). Enzymatic analysis of these acids is considered a routine method by many wineries.

Thus, there is undoubtedly interest in finding an enzymatic method for FA analysis since this would make it easier for wineries to determine it. To our knowledge, there are only two commercially available enzymatic kits for fumaric acid quantification. However, both are designed for very low FA concentrations because they are designed to estimate FA in cells and biological tissues (BioVision, 2013; Sigma-Aldrich, 2012) and are therefore not valid for estimating FA in wine. In addition, their manufacturers provide no information about their action mechanism.

The aim of this work is therefore to develop an enzymatic method for FA analysis in wines to encompass a wide range of concentrations (0–1.5 g/L) around the maximal dose of FA (0.6 g/L) authorised by the OIV (Resolution OENO-TECHNO 15-581A) (OIV, 2021).

## MATERIALS AND METHODS

### 1. Chemicals and equipment

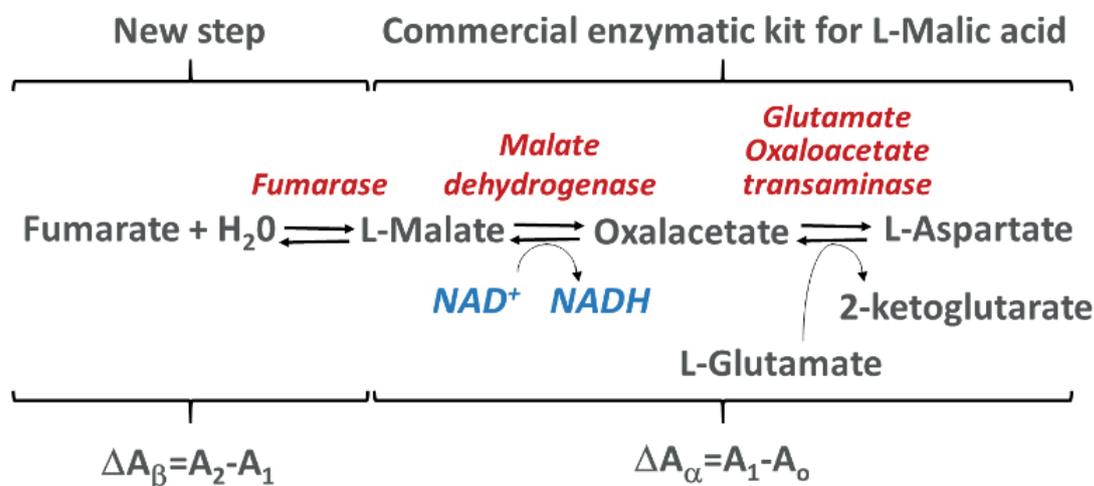
Absolute ethanol (96 % vol.), sodium hydroxide ( $\geq 98\%$ ), L-tartaric acid (99.5 %) and L-glutamate (99 %) were purchased from Panreac (Barcelona, Spain). Sodium carbonate anhydrous (99.5 %) was purchased from Fluka BioChemika (Buchs, Switzerland). The commercial kit for L-malic acid quantification was provided by r-Biopharm® (Art. No: 10139068035, Darmstadt, Germany). L-malic acid and fumarase from porcine heart were provided by Sigma-Aldrich (REF: F1757-2.5KU, Sigma-Aldrich, Madrid, Spain). Water was ultrapure Milli-Q quality (Millipore, Bedford, MA, USA). Centrifugations were done using a Biofuge Primo centrifuge (Heraeus, Hanau, Germany). All the spectrophotometric measures were analysed with a Helios Alpha UV-vis spectrophotometer (Thermo Fisher Scientific Inc., Waltman, MA, USA) using plastic semi-micro cuvettes of 10 mm path length (Greiner Bio-One, Madrid, Spain).

### 2. Action mechanism of the enzymatic method

The proposed enzymatic method is based on the classic kit for L-malic acid quantification with the inclusion of a new step catalysed by the

fumarase enzyme (EC 4.2.1.2). This enzyme, which participates in the Krebs cycle, reversibly transforms fumaric acid into L-malic acid, thus allowing the determination of fumaric acid by using it in connection with a classic L-malic acid kit.

Figure 1 shows its action mechanism. Briefly, when L-malate dehydrogenase (L-MDH) is added to the medium containing the wine solution at an appropriate dilution, the oxidised form of the coenzyme nicotinamide adenine dinucleotide ( $\text{NAD}^+$ ) and the buffer, L-malic acid (MA) is transformed into oxaloacetate (OAA) and  $\text{NAD}^+$  in its corresponding reduced form (NADH). Since this reaction is reversible, it is necessary to transform the OAA into L-aspartate (L-Asp) by the action of the enzyme glutamate oxaloacetate transaminase (GOT) in the presence of a great excess of L-glutamate (L-Glu) to guarantee that the equilibrium is completely displaced. This step ensures that the production of NADH will be stoichiometric with the initial MA, making it possible to quantify it by measuring the increase in absorbance at 334, 340 or 365 nm. If fumarase (FUM) is added once this reaction is completely finished, the FA is transformed into MA which is subsequently transformed first into OAA and then into L-Asp in accordance with the enzymatic reaction described in Figure 1. Thus, a second increase in the concentration of NADH is brought about which is stoichiometric with the initial FA concentration, making it possible to quantify it by determining a second increase in absorbance at 334, 340 or 365 nm.



**FIGURE 1.** Action mechanism of the enzymatic method for fumaric acid quantification.

**TABLE 1.** Summary of main parameters of the different matrix media used.

Media	Characteristics	Blending	Origin
Model wine synthetic solution	Ethanol content: 12.0% (v/v) Titratable acidity: 4.0 g/L pH: 3.5 adjusted with NaOH	-	None. Laboratory made
White wine	Ethanol content: 12.4 (v/v) Titratable acidity: 4.90 g/L pH: 3.23	35% Muscat of Alexandria 35% Muscat blanc à petit grans 15% Chardonnay 15% Xarel·lo 80% Tempranillo	Experimental vineyard and winery of University Rovira i Virgili (AOC Tarragona; Spain); Vintage 2020
Red wine	Ethanol content: 12.9 (v/v) Titratable acidity: 4.40 g/L pH: 3.37	10% Grenache Noir 8.5% Syrah 1.5% Pinot Noir	Experimental vineyard and winery of University Rovira i Virgili (AOC Tarragona; Spain); Vintage 2018
White Grape juice	Sugar content: 22.3 °Brix Titratable acidity: 3.45 g/L pH: 3.84	100% Muscat of Alexandria	Experimental vineyard and winery of University Rovira i Virgili (AOC Tarragona; Spain); Vintage 2020

### 3. Matrix media solutions used for validation of the proposed enzymatic method

Four different matrix media were used to verify the effectiveness of the proposed enzymatic method: a model wine synthetic solution, a white wine, a red wine and a white grape juice. The characteristics of these matrix media are summarised in Table 1. All were centrifuged at 7800 rpm (RCF: 4470  $\times$  g) for 10 min to avoid possible interferences of turbidity on absorbance reading. All these matrix media were used as solvents for preparing standard solutions of fumaric acid at the following concentrations: 0, 0.3, 0.6, 0.9, 1.2 and 1.5 g/L.

The model wine solution and the red wine were prepared and supplemented or not with L-malic acid (1.2 g/L). The white wine and the white grape juice were not supplemented with MA because they already contained appreciable amounts of this acid. Given the low solubility of fumaric acid, it was necessary to keep stirring for at least one hour to guarantee its complete dissolution. The model wine synthetic solution containing 0.6 g of fumaric acid per litre was prepared with increasing concentrations of L-malic acid (0, 2, 4, 6 and 8 g/L) to determine how the presence of MA might affect the quantification of FA.

### 4. Protocol for fumaric acid quantification using the proposed enzymatic method

All the samples were diluted 1:10 with ultrapure Milli-Q quality water to adjust the concentration of both acids (MA and FA) to the sensibility of the commercial L-malic kit. The analytical procedure is an adaptation of that described by the manufacturer of the commercial L-malic enzymatic kit. All absorbance measurements were performed at 365 nm because at this wavelength the molar absorptivity coefficient of NADH is suitable for working with only a dilution of 1:10. Working at 334 or 340 nm would require unnecessarily greater dilutions. Table 2 shows the adapted final protocol in which there are two options depending on the L-malic concentration of the sample.

Solutions 1 to 4 are those corresponding to the commercial kit. Specifically, solution 1 is a glycylglycine buffer adjusted at pH 10 and containing L-glutamic acid (100 mM). Solution 2 contains NAD<sup>+</sup> (55 mM) dissolved in water. Solution 3 contains the enzyme GOT (400 enzymatic units/mL). Solution 4 contains L-MDH (6000 enzymatic units/mL). Finally, the fumarase solution contains 1120 enzymatic units/mL. Solution 1\* is an L-glutamic acid solution (300 mM) that has to be used only when the L-malic acid concentration of the sample is greater than 4 g/L.

**TABLE 2.** The protocol followed for fumaric and L-malic acid quantifications.

	Pipette into cuvette	Volume ( $\mu\text{L}$ )	
		Conventional assay	Assay for wines with $<4$ g/L of MA
Solution 1	L-Glutamic acid (100 mM) in glycylglycine buffer, pH 10	400	400
Solution 1*	L-Glutamic acid (300 mM) in $\text{Na}_2\text{CO}_3$ at 2 %	0	200
Solution 2	$\text{NAD}^+$ solution (55 mM)	80	150
Solution 3	GOT (400 U/mL)	4	4
Sample	Wine, blank (water) or standard (sol. 5)	40	40
Water	Ultrapure Milli-Q quality water	360	90
	Mix, read absorbance at 365 nm after 5 minutes ( $A_0$ )		
Solution 4	L-MDH (6000 U/mL)	4	4
	Mix, read absorbance at 365 nm after 5 minutes ( $A_1$ )		
Fumarase	FUM (1125 U/mL)	5	5
	Mix, read absorbance at 365 nm after 50 minutes ( $A_2$ )		

The final concentrations of MA and FA can be obtained using the following equations (Figure 2) that were obtained bearing in mind the volume of the sample used for the measurement, the final volume in the cuvette, the dilution factor used in the sample and the molar absorptivity coefficient of NADH at 365 nm.

### 5. Statistical analysis

All the assays were performed analysing five replicates and are expressed as the arithmetic average  $\pm$  standard deviation. The limit of detection (LoD) and limit of quantification (LoQ) in the case of regression lines, instrument response  $y$  (in  $y = a + bx$  expression), is assumed to be linearly related to the standard concentration  $x$ . Thus each of the LoD and LoQ assays can be calculated as a division of the standard deviation by the slope of each regression line, per three and ten times, respectively (Shrivastava and Gupta, 2011).

## RESULTS AND DISCUSSION

### 1. Linearity of the proposed method in the different media

Figure 3 shows the results obtained for MA and FA concentrations in the different studied matrix media: model wine synthetic solution without L-malic acid supplementation (Figure 3A), model wine synthetic solution with L-malic acid supplementation (Figure 3B), white wine (Figure 3C), red wine without L-malic acid supplementation (Figure 3D), red wine with L-malic acid supplementation (Figure 3E) and white grape juice (Figure 3F).

These graphs show that in all the studied matrix media the concentration of FA presented satisfactory linear regression coefficients ( $r^2$  between 0.9985 and 0.9999). Moreover, the slopes of the regression lines were very close to 1 (between 0.9391 and 1.0447), which indicates that the recovery of the added FA was nearly complete by this method.

$\Delta A_\alpha = (A_1 - A_0)$ $[\text{L-Malic acid}] \text{ (g/L)} = 8.756 \times \Delta A_\alpha$	$\Delta A_\beta = (A_2 - A_1)$ $[\text{Fumaric acid}] \text{ (g/L)} = 7.622 \times \Delta A_\beta$
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**FIGURE 2.** Equations for L-malic and fumaric acid quantification, following the proposed method at wavelength 365 nm of absorbance.

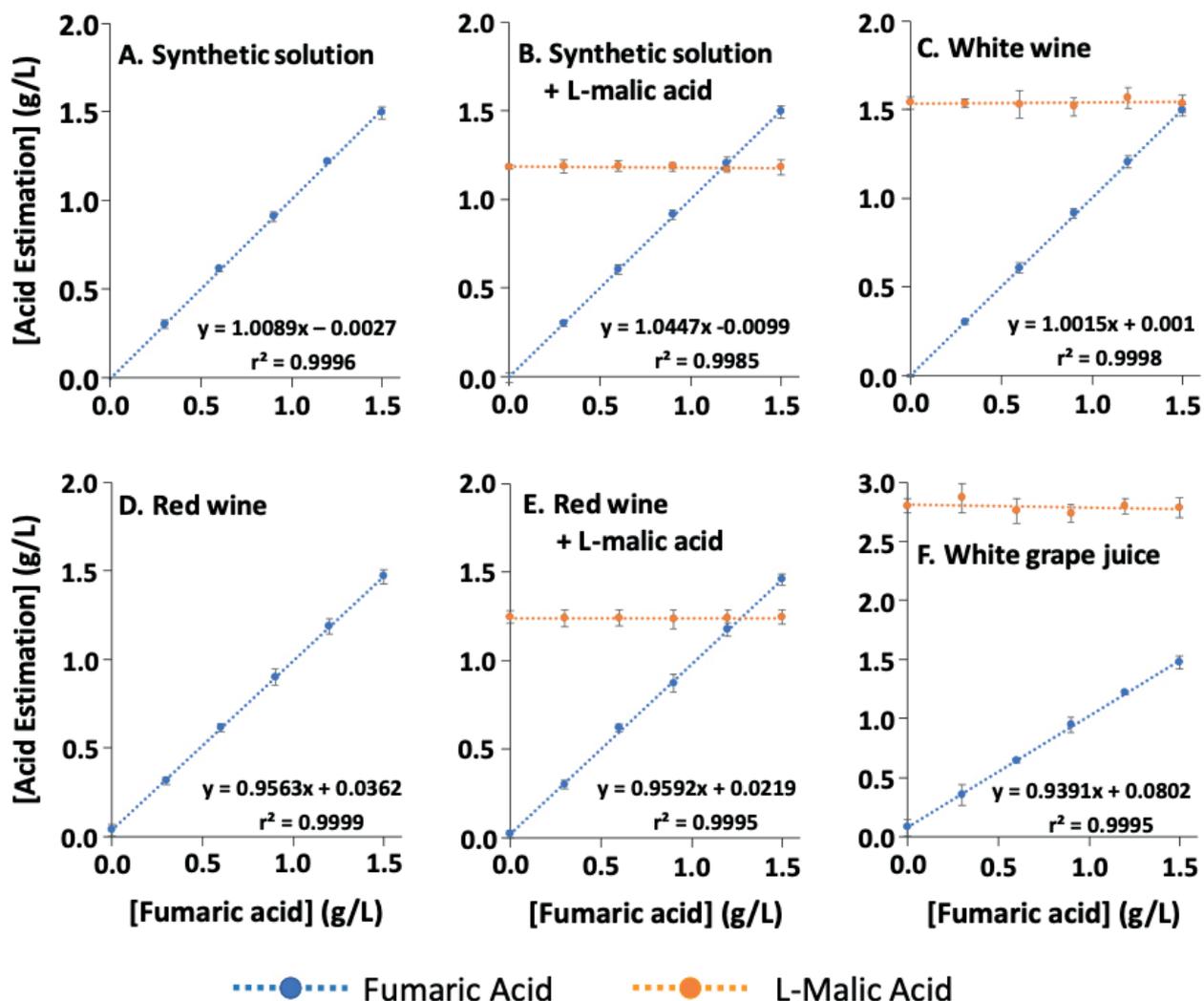


FIGURE 3. Fumaric and L-malic acid measurements in different matrix media solutions.

In the case of the white wine, the white grape juice, the synthetic solution and the red wine supplemented with MA, FA was quantified with satisfactory precision as can be seen in Figures 2B, 2C, 2E and 2F. These results, therefore, confirm that MA and especially FA can be successfully analysed using the proposed new enzymatic method.

## 2. Limit of determination and limit of quantification

The limit of determination (LoD) and the limit of quantification (LoQ) for FA determination in the different media were evaluated. LoD ranged from 0.012 to 0.043 g/L, while LoQ values ranged from 0.038 to 0.142 g/L. In general, these values were minimal in the case of the synthetic solution, followed in increasing order by the white wine, red wine and white grape juice. It, therefore,

seems that the presence of phenolic compounds in the case of red wine and of sugars in the case of grape juice could slightly increase the LoD and LoQ values. Certainly, it can be said that the proposed enzymatic method for FA analysis can be applied satisfactorily in all the studied media.

TABLE 3. Limits of detection and limits of quantification for the proposed method in each matrix media solution.

Matrix media	LoD (g/L)	LoQ (g/L)
Synthetic solution	0.012	0.038
Synthetic wine + MA	0.016	0.054
White wine	0.016	0.054
Red wine	0.027	0.090
Red wine + MA	0.031	0.102
White grape juice	0.043	0.142

### 3. Influence of L-malic acid concentration on fumaric acid analysis

The proposed enzymatic method for FA analysis needs to previously determine L-malic acid. We, therefore, considered the possibility that some concentrations of some of the reagents of the L-malic acid kit such as  $\text{NAD}^+$  and L-Glu may not be enough to work with wines with very high concentrations of MA. If the MA concentration is too high, there could be a risk that the  $\text{NAD}^+$  concentration would decrease excessively

and thus be insufficient for the subsequent FA analysis. Similarly, when the MA concentration is too high there is also a risk that the remaining L-Glu concentration may not be enough to completely displace the OAA/ASP equilibrium towards the formation of ASP. We, therefore, performed an assay to determine the influence of MA concentration on FA analysis. For this, a synthetic solution with 0.6 g/L of FA and increasing MA concentrations (0–8 g/L) was used. Figure 4 shows the results obtained.

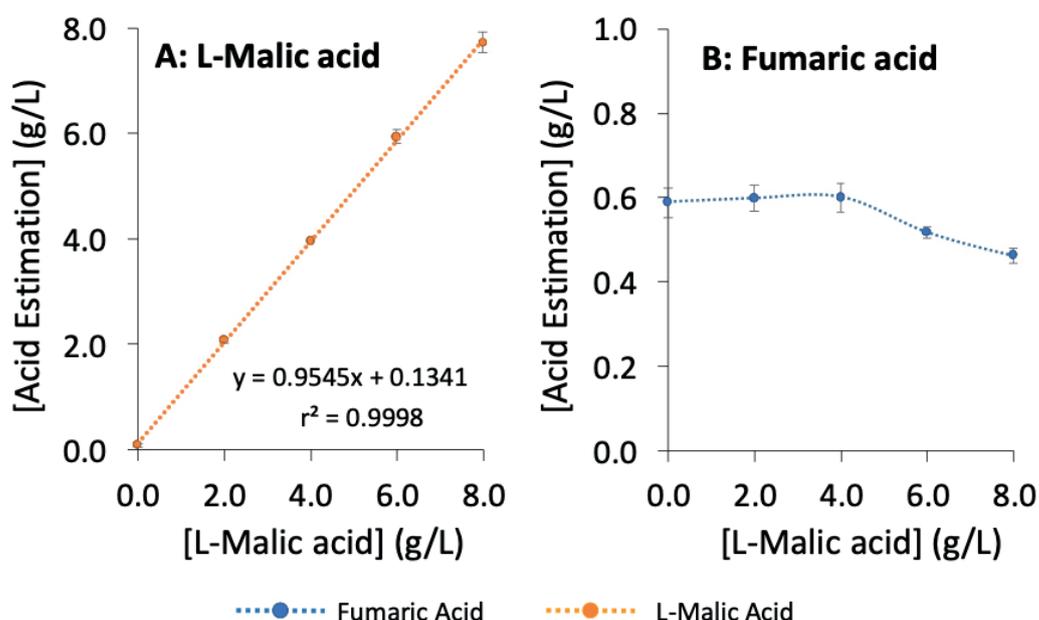


FIGURE 4. Influence of L-malic acid concentration on the measurement of fumaric acid in synthetic media.

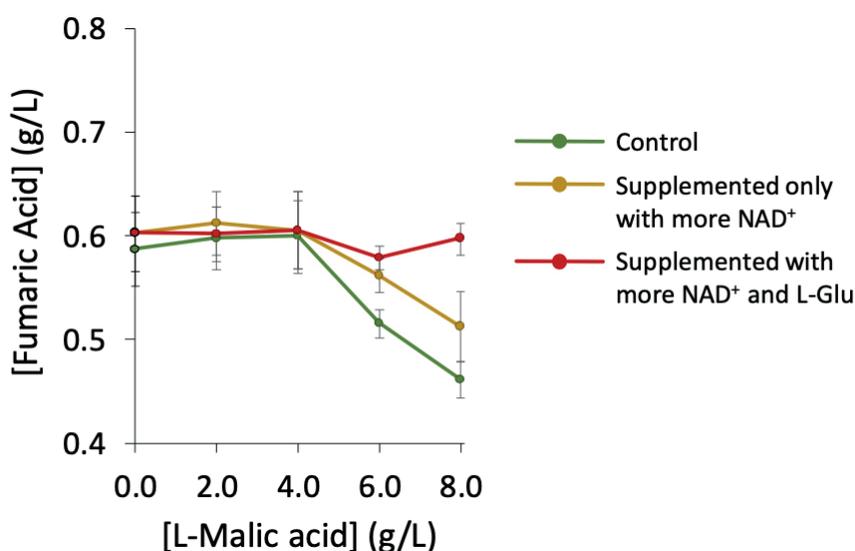


FIGURE 5. Improving the method of fumaric acid quantification for samples with L-malic concentrations higher than 4 g/L

The results show that when MA is present at concentrations above 4 g/L, the FA quantification tends to decrease. This data confirms that the expected depletion effect on NAD<sup>+</sup> and L-Glu concentrations caused by a high MA concentration really does affect the FA analysis. This limiting concentration of MA, 4 g/L, may be considered very high but is not infrequent in wines from cold climates. To solve the problem, the possibility could be considered of increasing the dilution factor of the sample, but this would probably affect the precision of the method. For that reason, we carried out another two assays. The first was performed increasing only the NAD<sup>+</sup> concentration (adding 150 µL of solution 2 instead of 80 µL). The second was performed by similarly increasing the NAD<sup>+</sup> concentration but also increasing the L-glutamic acid concentration by adding 200 µL of solution 1\*. In both assays, the volume of water was reduced to achieve the same final volume in the cuvette. Figure 5 shows the results obtained.

The results indicate that when only the NAD<sup>+</sup> concentration was increased the FA quantification improved, but not sufficiently. In contrast, when the L-Glu concentration was also increased, the method makes it possible to satisfactorily quantify the FA even at a very high L-malic concentration.

## CONCLUSION

The proposed new enzymatic method for fumaric acid analysis has been shown to be efficient and sufficiently robust in different media (synthetic solution, white wine, red wine and white grape juice). By including just one new step (the addition of fumarase enzyme) to the commercial kit process, it is possible to efficiently determine fumaric acid concentrations in a range between 0 and 1.5 g/L, which amply covers the proposed maximal dose of this acid (0.6 g/L) authorised by the OIV. The method also makes it possible to determine L-malic acid, since the first steps are precisely those used when analysing this acid using the commercial kit. However, if the L-malic concentration of the sample is very high ( $\geq 4$  g/L), the precision of the method decreases because the excess of L-malic acid causes a depletion of NAD<sup>+</sup> and L-glutamic acid concentrations. For that reason, a modified protocol is also proposed for wines that have more than this limiting L-malic acid concentration. This modified protocol allows fumaric acid to be determined in samples that are very rich in L-malic acid because it compensates for the depletion by adding supplementary amounts of NAD<sup>+</sup> and L-glutamic acid.

Furthermore, this new method is relatively fast (1 hour), easy to use and inexpensive, especially when compared with the official HPLC method. Bearing in mind the price of a commercial L-malic acid kit and that of fumarase, the final cost of fumaric acid determination through this method is around 5 €/sample.

For all these reasons we believe that this new enzymatic method can be proposed as a routine analysis for fumaric acid in wineries.

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