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A simple and effective method for the enzymatic analysis of fumaric acid in wine

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The use of fumaric acid (E297) at a maximum dose of 0.6 g/L to inhibit malolactic fermentation in wines has been recently approved by International Organisation of Vine and Wine (OIV)[1] and by the European Union (EU)[2]. This article describes a recently published enzymatic method to determine simultaneously L-malic acid and fumaric acid[3]. This method, which is very simple and efficient, 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.

Chemicals and equipment needed

- A spectrophotometer capable of working in the ultraviolet (365 nm).
- Plastic semi-micro cuvettes of 10 mm path length.
- A benchtop centrifuge and suitable centrifuge tubes.
- Usual volumetric material and automatic pipettes of 10-50, 50-200 and 200-1000 µL.
- A commercial L-malic enzymatic kit (r-Biopharm® (Art. No: 10139068035, Darmstadt, Germany).
- Fumarase from porcine heart (REF: F1757-2.5KU, Sigma-Aldrich, Madrid, Spain).
- Sodium L-glutamate 1-hydrate (99 %) (CAS: 6106-04-3).

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 involving catalysation by the fumarase enzyme (EC 4.2.1.2). This enzyme 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 is added to the reaction media containing the sample of wine, the buffer, the coenzyme nicotinamide adenine dinucleotide (NAD^+), an excess of L-glutamate (L-Glu) and the enzyme glutamate oxaloacetate transaminase (GOT), L-malic acid (MA) is completely transformed into oxaloacetate (OAA) and NAD^+ in its corresponding reduced form (NADH). Doing this means 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 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 above. 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 365 nm.

Protocol for fumaric acid quantification using the proposed enzymatic method

All the samples were centrifuged and 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. 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

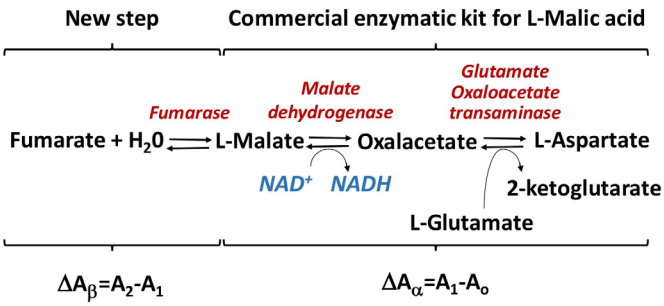

FIGURE 1. Action mechanism of the enzymatic method for fumaric acid quantification.

TABLE 1. Protocol followed for fumaric and L-malic acid quantifications.

Pipette into cuvette		Volume (µL)	
		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 Na_2CO_3 at 2%*	0	200
Solution 2	*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 (6,000 U/mL)*	4	4
Mix, read absorbance at 365 nm after 5 minutes (A_1)			
Fumarase	*FUM (1,125 U/mL)*	5	5
Mix, read absorbance at 365 nm after 50 minutes (A_2)			

final protocol in which there are two options depending on the L-malic concentration of the sample. The analytical procedure is an adaptation of that described by the manufacturer of the commercial L-malic enzymatic kit.
Solutions 1 to 4 are those corresponding to the commercial kit. The fumarase solution contains 1,120 enzymatic units/mL. Solution 1* is a 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.

The final concentrations of MA and FA can be obtained using the following equations 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.

$\Delta A_\alpha = (A_1 - A_0)$	$\Delta A_\beta = (A_2 - A_1)$
[L-Malic acid] (g/L) = 8.756 x ΔA_α	[Fumaric acid] (g/L) = 7.622 x ΔA_β

Linearity of the proposed method in the different media

Figure 2 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 2A), model wine synthetic solution with L-malic acid supplementation (Figure 2B), white wine (Figure 2C), red wine without L-malic acid supplementation (Figure 2D), red wine with L-malic acid supplementation (Figure 2E) and white grape juice (Figure 2E).

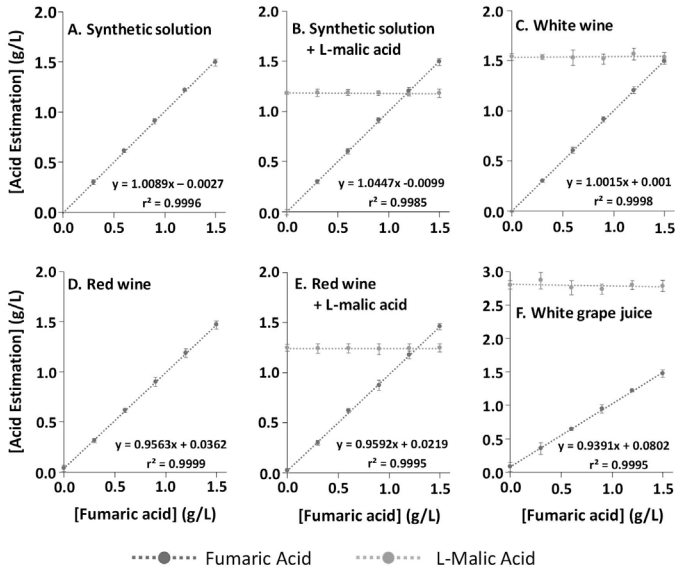

FIGURE 2. Fumaric and L-malic acid measurements in different matrix media solutions.

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. These results therefore confirm that FA can be successfully analysed using the proposed new enzymatic method.

Conclusions

The proposed enzymatic method for fumaric acid analysis was shown to be efficient and sufficiently robust in different media (synthetic solution, white wine, red wine and even 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 authorised dose of this acid (0.6 g/L). 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 (≥ 4 g/L), it will be necessary to modify this method by increasing the L-glutamate and NAD^+ concentrations.
Furthermore, this new method is relatively fast (1 hour), easy to use and inexpensive (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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Sources: *Sourced from the research article: "New enzymatic method for estimating fumaric acid in wines" (Oeno One, 2021).*

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