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ORIGINAL RESEARCH PAPER

DEVELOPMENT AND VALIDATION OF AN ENZYMATIC METHOD FOR THE DETERMINATION OF CITRIC ACID IN WINES

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Abstract

Citric acid is a crucial element in wine, affecting its flavor, acidity, and overall quality. The main objective of this study was to develop and validate an enzymatic technique for quantifying citric acid in wine, as a basis for assessing wine quality. The technique uses specific enzymes to facilitate the transformation of the citric acid into measurable compounds, providing a precise and reliable approach for quantification. The performance of the method was assessed using several parameters to ascertain its precision, accuracy, and linearity over the range of citric acid concentrations commonly present in wine. The method was linear in the citric acid measurement range of 23 mg/L - 380 mg/L with a correlation coefficient R^2 of 0.9941. The limit of detection was determined to be 19 mg/L, while the limit of quantification was established at 37 mg/L. The recovery was in the range 92.29-103.66% while the relative standard deviation was less than 2%. The method was additionally evaluated against standard reference techniques, confirming its appropriateness for routine analysis in wine quality assessment. This enzymatic approach is a reliable and economical option for quantifying citric acid levels in wine, facilitating more accurate and efficient oversight of wine production operations.

Keywords: wine, citric acid, enzymatic method, linearity, repeatability, reproducibility, detection limit, quantification limit

Introduction

Wine is a globally consumed fermented beverage, with many years of tradition. Establishing the authenticity of wine is crucial for food quality and safety (Sass-Kiss

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et al., 2008). Organic acids are essential for the stability of wine. The analysis of wines is essential for quality control and for monitoring the evolution of acidity throughout various stages of winemaking, from grape juice to alcoholic fermentation and wine stabilization, as significant changes in wine can be identified through variations in acid content (Scutarasu *et al*., 2021). Acidity is an important component of the winemaking process. Various acids exist, either in free or in the form of complexes, including the organic acids originating from grapes (malic, tartaric, and citric acids) as well as others (succinic, acetic, and lactic acids) that result from diverse fermentation processes. The fermentation of wine facilitates the transformation, disappearance, or new formation of various acids (Payan *et al.*, 2023).

The concerns and potential hazards associated with synthetic antimicrobials and antioxidants raised the consumers' interest in natural and safe alternatives (Perumalla and Hettiarachchy, 2011). Consequently, it is essential to assess the levels of organic acids in wine samples, not only for food quality control, particularly regarding wine, but also due to their advantageous effects on human health. According to a study by Tang *et al.* (2013), citric and malic acids have considerable preventive effects on the myocardium and influence ischaemic lesions. Nagai *et al.* (2010) revealed that oral administration of citric acid enhances ketosis and safeguards against the onset of diabetes in an animal model of type 1 diabetes. Bortz *et al.* (2006) indicated that short-chain organic acids (succinic acid, acetic acid, citric acid, lactic acid, malic acid, glutamic acid, and their salts) serve as beneficial adjuncts to iron absorption.

The citric acid (citrate; food additives E330–E333 as per EU regulation EC/1333/2008) is utilized in a diverse array of foods and beverages, including fruit juices, carbonated soft drinks, beer, bread, confections, and dairy or meat products, owing to its superior acidifying, antioxidant, stabilizers and preservative attributes. Additionally, it is utilized in the wine production, with an acceptable upper limit of only 1 g/L, as stipulated by the Organisation Internationale de la Vigne et du Vin (OIV) code of practice (OIV, 2015). Moreover, the OIV and the EU recommend the use of citric acid for correcting the deficient acidity of wines, the condition being that the final wine should not contain more than 1 g/L. The rules for the application of the Law on Vine and Wine in the system of common organization of the wine market no. 164/2015 allow correction by adding citric acid; within the maximum limit of 2.5 g/L, the total acidity expressed in tartaric acid of the wines must not be higher than 1 g/L (OIV, 2015).

Citric acid is a monohydroxy tricarboxylic acid found in grapes, must, and wine. It is formed in the roots of the vine, from where it migrates into the grapes, and only a small part of the grapes is also formed in the leaves. The citric acid accumulates in grapes in lower amounts, ranging from 0.1 to0.8 g/L in grape must, compared to the other acids such as tartaric and malic acids. Botrytized (molded) grapes contain higher amounts of citric acid, sometimes exceeding 1.5 g/L of must. During the ripening period of the grapes, the citric acid content remains stable or even registers a slight increase. Therefore, citric acid is the most biochemically stable acid in wine

(Payan *et al.*, 2023). Citric acid is used to invert sugar (sucrose), which is added to the must in autumn when the grapes do not accumulate the necessary amounts of sugar. It is also used to correct the deficient acidity of wines, within the maximum limit of 2.5 g/L total acidity, expressed in tartaric acid. Citric acid binds the iron (Fe^{3+}) in wine in the form of a soluble complex anion (ferro citric). Therefore, in oenological practice, the addition of citric acid is used in white wines to prevent ferric cassation at a dose of 100 mg/L. It is added in the final phase of wine conditioning (Tsegay, 2020).

The changes in compound concentrations among different wine varieties may affect the interactions between volatile and nonvolatile substances in several ways. Consequently, the comparison of organic acid concentration among different wine samples should not rely solely on statistical data, but must also encompass sample preparation and analytical methods. Validated methodologies are essential to guarantee precise compositional data for regulatory compliance verification or to support dietary recommendations (Robles *et al.*, 2019). For the separation, detection, characterization, and quantification of citric acid in wines, the following procedures may be employed: spectrophotometric methods, enzymatic approach, capillary electrophoresis, high-performance liquid chromatography, gas chromatography, coupled to mass spectrometry, and infrared spectroscopy (Mato *et al.*, 2005). Furthermore, the International Organisation of Vine and Wine (OIV, Paris, France) regulated the prescribed methodologies for organic acid analysis. The specified standard techniques are evaluated and modified annually (Zeravik *et al.*, 2016).

The main objective of this study was to validate the enzymatic method for quantifying citric acid in wines, and to establish the performance parameters of the method in view of its use to identify possible frauds in the analyzed wine samples.

Materials and methods

Reagents

Ready-made reagents contained in the Biosystems enzyme kit (BioSystems S.A. Costa Brava, Barcelona, Spain) were used: citric acid enzyme kit; anhydrous citric acid p.a. for the preparation of working standards; tartaric acid; 1N sodium hydroxide; 1:1 hydrochloric acid; polyvinylpyrrolidone. Pyridine (99%) and acetic anhydrite (97%) were provided by Sigma Aldrich (Steinheim, Germany). All chemicals were of analytical grade.

Samples preparation

For the determinations, certified reference material - white wine samples provided by Biosystems (BioSystems S.A. Costa Brava, Barcelona, Spain - CRM) was used as a matrix. CRM was provided with three different citric acid concentrations: i) 65 mg/L with expanded uncertainty of 13 mg/L; ii) 275 mg/L with expanded uncertainty of 70 mg/l; and iii) 633 mg/L mg/L with expanded uncertainty of 95mg/L.

The method for determining the citric acid in a wine sample is based on the reaction with acetic anhydride in a basic medium, which forms a product whose maximum absorbance is read in a spectrophotometer at a wavelength of 363 nm.

A number of 10 samples of wines (4 white, 4 red, 2 rose) found on the market and produced in Vrancea county, Romania, were used to evaluate the enzymatic method in comparation with the reference method.

Apparatus

The occurring reactions were monitored on a BIOSYSTEMS Y15 automatic enology equipment (BioSystems S.A. Costa Brava, Barcelona, Spain), equipped with a thermoregulation system. Kern &Shon GMBH class E2 standard weights with a calibration certificate and a POLYSCIENCE thermostatic chamber with a calibration certificate were also used.

Reference method of citric acid determination

The reference method for citric acid content in a wine sample is based on the reaction with acetic anhydride in a basic medium, which forms a product with a maximum absorbance at 363 nm. Briefly, 25 mL of the wine sample was treated with 0.1g activated carbon powder and after 5 minutes it was filtered. Then, a volume of 10 mL of clear wine was mixed with 2 mL of pyridine (99%) and 1 mL of acetic anhydrite (97%). A volume of 25 mL of distilled water was added in the mixture and, after homogenization, the absorbance was measured at 363 nm using a Libra S22 UV-VIS spectrophotometer (Biochrom, Cambridge, UK). The citric acid content was determined using the citric acid standard curve (0.1–10 mg/L).

Enzymatic method of citric acid content determination

The method is based on the transformation of citric acid (citrate) into oxaloacetate and acetate through a reaction catalyzed by a specific enzyme - citrate lyase (CL). In the presence of the enzymes malate dehydrogenase (MDH) and lactate dehydrogenase (LDH), the oxaloacetate and the decarboxylation derivative pyruvate are reduced to L-malate and L-lactate by the enzyme nicotinamide adenine dinucleotide (NADH) reductase. The amount of NADH oxidized to NAD+ by the mentioned reactions is proportional to the amount of citric acid present in the sample. The oxidation of NADH is quantified by the decrease in the absorbance value read at 340 nm using Libra S22 UV-VIS spectrophotometer (Biochrom, Cambridge, UK). The values obtained were expressed as mg/L citric acid.

Parameters used for method validation

The purpose of validation is to establish that a specified analytic system yields valuable and reproducible findings for a particular attribute.

To validate the method for citric acid determination in wines, the following performance criteria were checked: selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, recovery, precision, repeatability, reproducibility (internal/external), robustness, and uncertainty of measurement.

Selectivity is a performance parameter of the applied analytical method, showing the ability of the method to differentiate and measure different analytes, in the presence of other components that are normally present in the sample.

In this study, for selectivity evaluation of the method, the presence of the tartaric acid in the samples was considered. Tartaric acid is a compound with a chemical

structure similar to citric acid. A number of 10 quantitative determinations were performed using the enzymatic method for determining citric acid from a certified reference white wine sample, having a content of 290 mg/L citric acid. Other 10 determinations of citric acid by the enzymatic method from the same certified reference white wine sample but in this case enriched with 10 g/L tartaric acid were done. The average of the results before enrichment ($Mx=1/n\sum Mxi$), the average of the results after enrichment ($My=1/n\sum Myi$), the difference of the means (Md), the standard deviation (Sd) of the trials, and the z score = (Md/Sd) were calculated.

Linearity is the ability of a method to provide results directly proportional to the concentration of the analyte in the measured sample. The Lambert-Beer Law was applied to the spectrophotometer measurements. Experimentally, linearity was verified using the certified reference white wine with a concentration of 633 mg/L citric acid, diluted to the value corresponding to the upper limit of the measurement range. The calibration curve was automatically generated by the analyzer by performing sample dilutions at 6 points of different concentrations. The determined concentration within ± 15 % of expected concentration and the correlation coefficient (R^2) being ≥ 0.990 were accepted.

Limit of Detection and Limit of Quantification. LOD is the smallest amount of a compound that can be detected, identified, and not necessarily quantified using the equipment and methodology in question. The theoretical LOD (declared by the enzyme kit) was 11 mg/L citric acid, detected at a wavelength of 340 nm. LOQ represents the smallest amount of analyte in the sample that can be detected and quantified with acceptable repeatability and accuracy.

It was verified if the LOD measured under the experimental conditions of the laboratory is similar to that declared by the producer. Thus, a wine sample (CRM white wine diluted to a concentration of 11 mg/L citric acid) was analyzed repeatedly (10 times). The mean of the tests (X average), the standard deviation of the tests (S) and LOD = X average +3S were calculated. After the calculation of LOD value, the method was used to evaluate a standard solution with a concentration of 19 mg/L citric acid. Thus, the analysis was repeated 10 times, and the average of the tests (X average), the standard deviation of the tests (SD), relative standard deviation (RSD%), and recovery (%) were calculated.

LOQ was calculated after measuring citric acid concentration for 10 times using certified reference white wine diluted to a concentration of 37 mg/L citric acid. The mean of the tests (X average), the standard deviation of the tests (S) were estimated and the LOQ = X average + 10S was calculated.

Accuracy, recovery, and precision

Accuracy/Precision error is the difference between the average of the results of a measurement and an accepted reference value. The accuracy/correctness of measurements can also be verified by interlaboratory comparisons.

Correctness can be defined as the degree of agreement between the values obtained by the reference method and by the usual method, regardless of the loyalty errors of both methods.

In the situation of accredited laboratory, tests can be performed by two alternative methods (conformity with OIV-MA-ASI-12:R2005 CAP.5.3.3).

BIAS (D%): accuracy relative to the reference value

$$I = \text{accuracy/precision error} = X \text{ average} - X \text{ theoretical} \quad (1)$$

where X average is the arithmetic mean of a series of values obtained by measurement; X theoretical or X0 is the reference value or conventionally true value.

$$\text{BIAS (D\%)} = [(X \text{ average} - X \text{ target})/X \text{ target}] \times 100 \quad (2)$$

Certified reference white wine was used, and 10 measurements for the same sample were performed. The method meets the accuracy criterion required for the analyses performed when the values for D% are lower than those specified (OIV-MA-ASI-12:R2005).

Recovery represents the percentage of the true concentration of a substance, recovered during the analytical procedure. For the validation of the enzymatic method, a certified reference white wine fortified with anhydrous citric acid up to 1000 mg/L was used, to cover the entire working range. Ten enzymatic determinations of citric acid were performed on each sample, the recovery was calculated for each concentration, the average recovery for each concentration, and it was verified if it fell within the required range. The recovery was calculated as [(final concentration-initial concentration)/added concentration]. Recoveries within 90%–120 % are accepted.

Repeatability represents the degree of agreement between the results of successive measurements of the same measurement carried out under the same measurement conditions (same measurement procedure, same operator, same measuring instrument used under the same conditions, same place, within a short period of time).

Accuracy (fidelity) represents the degree of agreement or dispersion between the results obtained under conditions of repeatability or reproducibility and is expressed as the standard deviation.

The standard deviation of repeatability (RSD) is the standard deviation of the results obtained under repeatability conditions. It is a parameter that shows the dispersion of the results under repeatability conditions. The percentage expression of the standard deviation of repeatability (precision error) is the coefficient of variation RSD or CV and was calculated by formula 3:

$$\text{RSD (\%)} = \text{CV (\%)} = (Sr/Xm) \times 100 \quad (3)$$

where Xm is the average value of the analyte concentration; Sr is the standard deviation of repeatability.

The repeatability limit (R) is the value below which the difference between two determinations of the analysis obtained under repeatability conditions can be estimated, with a confidence level of 95%. Repeatability was calculated using the formula: $R = 2.8 \times Sr$.

A number of 10 enzymatic determinations of citric acid was performed on each sample (certified reference white wine having 633 mg/L citric acid and fortified sample with anhydrous citric acid up to 1000 mg/L), by the same analyst, using the same equipment, and under repeatability conditions. The standard deviation of repeatability (SD), the repeatability limits (R) and the coefficient of variation (RSD% or CV%) were calculated, and the following conditions were verified: $R_{lab} \leq R_{OIV}$ (R_{lab} is the repeatability calculated in the laboratory and R_{OIV} is the repeatability imposed by the OIV) and RSDr%< 9.

Internal reproducibility represents the degree of agreement between the results of measurements of the same determination performed under different measurement conditions (different analysts, measuring instrument used under different conditions, over a longer period of time).

The standard deviation of reproducibility (SD) was determined. Using these values, the reproducibility limit (R) was calculated, which shows whether the difference between repeated analyses on a sample, determined under reproducibility conditions, is significant with a confidence level of 95%. The percentage expression of the standard deviation of reproducibility, namely the coefficient of variation (RSD% or CV%), was also calculated. Reproducibility was calculated according to the formula: $R=2.8 \times Sr$

To evaluate the internal reproducibility, 10 enzymatic determinations were performed with certified reference white wine having 275 mg/L citric acid. The tests were performed on different days (monthly) by different analysts under reproducibility conditions, the standard deviation of reproducibility (SD), the reproducibility limit (R), and the coefficient of variation (RSD% or CV%) were calculated, and the inclusion in R_{lab} was verified: $R_{lab} \leq R_{OIV}$, and RSDr%< 9.

External reproducibility represents the degree of agreement between the results of measurements of the same measurement carried out under different measurement conditions (different laboratories, different analysts, different measuring instruments). External reproducibility is determined by participating in intercomparison exercises. The z-scores, displacement, and standard deviation from the external control were analyzed. The laboratory participated in 2024 in 2 exercises organized by Bipea France for the enzymatic determination of citric acid in several stages.

Robustness of the method represents the measure of the ability of an analytical procedure to remain unaffected by small but deliberate variations in method parameters and to provide indications of safety (reliability) during normal use. Experimentally, the robustness of the enzymatic citric acid determination method was demonstrated by modifying the pH of a reference material CRM (290 mg/L). The certified reference material had a pH of 3.42 and a citric acid concentration of 298.77 mg/L. By adjusting the pH to 0.7, the determined citric acid concentration was 318.02 mg/L, and by adjusting the pH to 11.28, the determined citric acid concentration was 307.99 mg/L. The pH adjustment was achieved by adding solutions of HCl and NaOH at concentration of 1N.

Uncertainty of measurement

Uncertainty of measurement is a useful parameter associated with the result of a measurement that characterizes the dispersion of values that can reasonably be attributed to the measurement. Combined uncertainty was calculated taking into consideration all sources of uncertainty. Furthermore, the extended uncertainty was calculated.

Statistical analysis

This study presents the mean and standard deviation derived from three examinations. Data normality and homoscedasticity were evaluated using Minitab 19 (Minitab Inc., PA, USA), subsequently followed by one-way ANOVA and Tukey's test to determine significant differences ($p<0.05$).

Results and discussion

Validation parameters

Selectivity

Selectivity refers to the capacity to accurately identify the target analyte among other analytes, matrices, or possibly interfering substances that may be present in the sample or matrix (Prakashan and Martin, 2024). Sensitivity represents the change in the response of a measuring instrument to the corresponding change in the stimulus (VIM1983) - the change in the analytical response to a small change in the analyte concentration. A method is considered selective if it can quantitatively determine only citric acid so that citric acid determination is not influenced by other compounds present in the sample.

According to OIV-MA-ASI-12:R2005, if z Score selectivity is 2 it can be considered that the influence of the added compound is negligible, and if z Score selectivity is ≥ 2, there is a 5% risk that the addition of the compound will influence the analysis result.

Therefore, the concentration determined before enrichment was Mx = 298.774 ± 9.516 mg/L citric acid, the concentration after enrichment was My = 292.250 ± 8.829 mg/L citric acid, and mean difference was Md = 11 ± 7.936 mg/L citric acid. The z score of 1.39, which is less than 2, indicates that the method is selective for citric acid, not being influenced by the presence of tartaric acid in the sample.

Linearity, Limit of detection and Limit of quantification

The linearity range, linear regression equation, limit of detection, and limit of quantitation are outlined in Table 1, demonstrating favorable linearity across the working concentration ranges.

The correct measurement of samples was performed in the measurement range in which the characteristic of the device is linear. The measuring range is the range of concentrations for which acceptable accuracy and precision can be achieved under linearity conditions. To evaluate the measurement range, the correlation coefficient, "R^2", was checked ($R^2 > 0.990$ good linearity; $R^2 > 0.997$ very good linearity).

Table 1. Overview of the optical and regression attributes of the proposed method

Parameters	**Citric acid estimation**
Linear dynamic range (mg/L)	23 - 380
Regression equation	y = 0.0069+0.0010x
Correlation coefficient (R^2)	0.9941
LOD (mg/L)	19
LOQ (mg/L)	37.05

In our study, the measurement range of citric acid was 65-1000 mg/L. The measurement range for citric acid, specified for the Biosystems enzymatic analyzer was 11-400 mg/L. All certified reference white wine samples with concentrations above the upper limit of measurement were diluted, and finally the citric acid concentration obtained was multiplied by the dilution applied to the respective sample. The calibration curve was y = 0.0069 + 0.0010x with linear correlation coefficient (R^2) of 0.9941. The method was linear in the citric acid measurement range of 23 mg/L - 380 mg/L.

The linearity of the citric acid measurement, defined as the detector's capacity to yield data directly proportional to their concentration within a specified range, was confirmed in the matrix-matched calibration curves. Consequently, the present method was considered linear in the reported concentration ranges for citric acid.

The sensitivity of the method was evaluated by assessing the LOD and LOQ. The limit of detection (LOD) and the limit of quantification (LOQ) are utilized to show the capacity of a method to identify and measure low concentrations of relevant material, respectively (Coelho *et al.* 2018). The LOD and LOQ values evaluated depend on the slope of the straight line.

For the calculated LOD, which was determined to be 19 mg/L for citric acid, the mean of the determined concentration was X average = 11.16 ± 2.588 mg/L citric acid while the LOD verification resulted in X average = 19.64 ± 1.651 mg/L citric acid.

The criteria for RSD% and recovery% were verified, with an RSD% < 10% and recovery% between 90 - 120%. In this case, the calculated RSD% was 8.41% and the recovery % was 103.4%, meeting the required standards.

Afterwards, the LOQ value of 37.05 mg/L was calculated. The mean of the determined concentration was X average = 37.94 ± 2.585 mg/L citric acid, the RSD % was 6.81%, and the recovery % was 104.22%.

According to OIV-MA-AS1-12: R2005 LOQ, two conditions should be valid:

$\frac{|LOQ-Xaverage|}{\frac{SD_{LOQ}}{\sqrt{10}}} < 10$ and $5 \times SD_{LOQ} < LOQ$

First condition $=\frac{|37.05-37.94|}{2.585/\sqrt{10}}=1.09<10$

Second condition = 5 × 2.585 = 12.92 < 37.05

The minimum detection limit for citric acid determined by the enzymatic method, demonstrated in the laboratory, was LOD = 19 mg/L citric acid, and the minimum quantification limit for citric acid determined by the enzymatic method, demonstrated in the laboratory, was LOQ = 37 mg/L citric acid. These limits have been demonstrated and verified according to OIV-MA-AS1-12:R2005. All these indicated the satisfying linearity within a wide linear range.

Consequently, the optimized method has suitable sensitivity for the analysis of citric acid present in low concentration.

Accuracy, recovery, and precision

The assessment of the method's accuracy is essential for maintaining analytical quality and can be conducted by various approaches: employing recovery assays, certified reference materials, and internal standards (Funk *et al.*, 1995; Long and Winefordmer, 1983). The accuracy was assessed by evaluating the efficacy of the kit procedure in recovering added analytes.

Accuracy (trueness) is the closeness between the value obtained by measurement and the theoretical expected value (ISO 3534-1:2006).

The trueness of the method was assessed through mean percentage recoveries, while precision was evaluated as the relative standard deviation (RSD) for both repeatability (intraday) and laboratory reproducibility (interday).

Our findings indicate that there is no significant variability in precision across different concentrations recorded on the same or different days.

The results obtained for accuracy are shown in Table 2. C (%) is the standard deviation of the repeatability (for accuracy estimation) of a reference material and it was established depending on the concentration of the analyte in the validation procedure or by OIV-MA-AS1-12:R2005. The results obtained fall within the respective requirements imposed by the laboratory accuracy (D%) < C (10%).

Table 2. Accuracy, spike recovery, and precision results for citric acid

Matrix	Spiked citric acid level	Mean value	SD %	Accuracy, (BIAS%, D%)	Recovery, (%)	Precision, (RSD%, CV%)
Low	65 mg/L	64.70	3.561	0.46	103.66	1.850
Mild	275 mg/L	262.80	3.676	4.44	92.63	1.399
High	633 mg/L	622.20	6.286	1.71	92.29	1.010
High	1000 mg/L	949.2	7.554	7.71	93.24	1.433

The inter- and intra-day accuracy and precision % relative standard deviation (RSD%) for each of the tested concentration of citric acid (65, 275, 633, 1000 mg/L) were found to be less than 9% which are within the OIV-MA-AS1-12: R2005 range.

Trueness (or bias) refers to the closeness agreement between the mean of an infinite number of replicated measured quantities and a reference value (Prakashan and Martin, 2024).

Recovery study was also performed to determine the accuracy of the method. Spiked recoveries are presented in Table 2. Good recoveries were obtained for citric acid that was consistently above 90%. For all determinations, at different concentration levels, the calculated recovery falls within the imposed requirements with an average of 97.08%.

The precision data demonstrated that the analyte exhibited acceptable precision at each tested level according to OIV-MA-AS1-12:R2005 for citric acid estimation.

The results of the recovery are presented in Table 2. These results meet the accepted validation requirements. Precision was tested on 10 replicated analyses of independent preparations of citric acid. The RSD values ranged from 1.850% to 1.010% indicating that the method was precise with a high degree of repeatability for citric acid (RSD < 2%). The recovery of citric acid from wines ranged from 92.29 and 103.66% confirming the accuracy of the separation and analysis conditions.

Repeatability

The repeatability limit value for the enzymatic determination of citric acid, according to OIV-MA-AS313-09, is as follows: for citric acid concentrations less than 400 mg/L, the repeatability limit (R_{OIV}) is 14 mg/L, and for concentrations higher than 400 mg/L, the repeatability limit (R_{OIV}) is 28 mg/L. The repeatability limits obtained in the laboratory must be $R_{lab} \leq R_{OIV}$.

The RSDr% limit established in the laboratory, according to OIV-MA-AS1-12:R2005 recommendations for the quantitative level at which the analysis was carried out (0.1 %) was RSDr% < 9.

The obtained repeatability results are shown below in Table 3.

Table 3. Repeatability data

Matrix	Spiked citric acid level	Mean value	SD %	CV, % (RSD)	Accuracy, % (D%)	R_{lab}	R_{OIV}
High Enriched with citric acid	633 mg/L	622.2	6.286	1.249	7.71	17.60	28
	1000 mg/L	949.2	7.554	1.433	6.76	21.15	28

For all determinations, at different concentration levels, the repeatability calculated in the laboratory was lower than the one imposed by the OIV and the coefficients of variation obtained under repeatability conditions reveal a repeatability precision within acceptable limits under our laboratory conditions.

Internal reproducibility

The results obtained are presented in Table 4. The reproducibility limit value for the enzymatic determination of citric acid, according to OIV-MA-AS313-09, is as follows: for citric acid concentrations less than 400 mg/L, the reproducibility limit (R_{OIV}) is 39 mg/L, and for concentrations greater than 400 mg/L, the reproducibility

limit (R) is 65 mg/L. The reproducibility limits determined in the laboratory should be: $R_{lab} \leq R_{OIV}$.

The limit established in the laboratory, according to OIV-MA-AS1-12:R2005 recommendations for the quantitative level at which the analysis was carried out (0.1%), was RSDr % < 9.

Table 4. Internal reproducibility data

Matrix	Spiked citric acid level	Mean value	SD %	CV% (RSD)	BIAS (D%)	R_{lab}	R_{OIV}
CRM	275 mg/L	265.8	11.77	4.56	2.86	32.96	39

External reproducibility

Two interlaboratory tests were performed during the year 2024. The calculated z scores were -0.4 and -0.2. Both values were included in $-2 \leq z \leq 2$. From the reports sent by the organizing company, it was found that the laboratory recorded a z score lower than 2 for the enzymatic analysis of citric acid in wines with different concentrations (Table 5).

Table 5. Laboratory results from the interlaboratory test

Analyte determination	Assigned value	Reported result	z-scores
Citric acid	0.18g/L	0.20g/L	-0.4
Citric acid	0.33g/L	0.32g/L	-0.2

Robustness

Robustness refers to the sensitivity to variation in one or more parameters. Changing parameters such as pH, temperature, reagents from different suppliers, operators should not have significant effects on the measurement results. Moreover, robustness is the ability of the method to lead to the same result when one or more working parameters are varied. The results obtained when checking the robustness of the method are presented in Table 6.

Table 6. Robustness data

Matrix	Spiked citric acid level	Mean value	SD, %	BIAS, % (D, %)	CV, % (RSD, %)
CRM pH 3.42	290 mg/L	298.774	9.516	3.026	3.185
CRM pH 0.7	290 mg/L	318.02	7.038	9.662	2.213
CRM pH 11.28	290 mg/L	307.994	11.337	6.205	3.681

Considering that the pH of the wine samples is acidic with values ranging between 2.8 and 4.5, by comparing the results obtained in the enzymatic determination of citric acid from the wine sample with a normal acidic pH with the results of the wine sample brought to a very acidic pH (0.7), it was found that no significant changes occured, so the enzymatic method was robust to pH changes.

Measurement of uncertainty

The estimated combined uncertainty was 0.0495. For CRM with a citric acid concentration of 65 mg/L, the calculated standard uncertainty was 65 x 0.0495 = 3.21. Further, the extended uncertainty was 2 × 3.21= 6.42.

Enzymatic method vs reference method of citric acid estimation in wines

The enzymatic method was applied to establish the citric acid profiles of ten commercially available (white, red, and rose) wines samples, as compared to the reference method (Table 7).

Table 7. Citric acid determination in wine samples

Samples	Citric acid determination (reference method), mg/L	Citric acid determination (enzymatic method), mg/L
White wine 1	257.66 ± 0.57*[a]	257.33 ± 0.57[a]
White wine 2	291.33 ± 0.57[a]	290.66 ± 0.57[a]
White wine 3	325.00 ± 1.00[a]	324.33 ± 0.57[a]
White wine 4	222.16 ± 1.25[a]	222.36 ± 1.12[a]
Red wine 1	115,76 ± 1.25[a]	114,86 ± 0.35[a]
Red wine 2	181,86 ± 0.85[a]	181,66 ± 0.30[a]
Red wine 3	231,24 ± 0.03[a]	231,92 ± 0.54[a]
Red wine 4	157,96 ± 0.65[a]	158,80 ± 0.20[a]
Rose wine 1	77.76 ± 0.57[a]	77.66 ± 0.57[a]
Rose wine 4	154.00 ± 1.00[a]	153.66 ± 0.57[a]

*standard deviation; The average values in the same row that do not have the same lowercase letters are significantly different ($p<0.05$).

For all tested samples, similar citric acid contents were found using both methods, the registered differences between results being lower than 1%.

Conclusions

This paper reports the findings of a study conducted to validate an enzymatic approach for the measurement of citric acid in white wine in terms of sensitivity, linearity, accuracy, precision repeatability, reproducibility, robustness, and measurement of uncertainty. The method is simple and displays good precision.

The values obtained for the validation parameters were considered satisfactory for the intended objective. The method was linear in the citric acid measurement range of 23 mg/L - 380 mg/L with a correlation coefficient $R^2 = 0.9941$. The coefficients of variation (CV) for repeatability ranged from 1.249 to 1.433%, CVs for

reproducibility ranged from 1.0 to 4.56%. The percentage recovery (92.29-103.66%) and the percentage relative standard deviation (%, RSD) was less than 2%. The robustness and the measurement of uncertainty results were excellent. The method is accurate and precise, with no interference from excipients. The methodology has been considered reliable for the characterization of red, white, and rose wines.

For all determinations, at different concentration levels, the repeatability calculated in the laboratory was lower than that one imposed by the OIV, and the coefficients of variation calculated under repeatability conditions revealed a repeatability precision within acceptable limits under the laboratory conditions.

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