Scientific Papers Series Management, Economic Engineering in Agriculture and Rural Development
Vol. 24, Issue 4, 2024
PRINT ISSN 2284-7995, E-ISSN 2285-3952

HPLC METHOD OPTIMISATION AND APPLICATION FOR THE ANALYSIS OF L(+) AND D(-) LACTIC ACID IN WINE - A WAY FOR ASSESSING WINE QUALITTY

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Abstract

Because of their antioxidant, antimicrobial and anti-inflammatory properties, there is an increasing interest in investigating the presence of organic acids in food. The analysis of organic acids in wine is of great importance, considering their role in organoleptic and aesthetic character. In the present study, a high-performance liquid chromatographic (HPLC) method was optimised and applied for rapid analysis of L-lactic acid and D-lactic acid in wine samples. The enantiomer separation was performed on a chiral column, using an aqueous solution of $CuSO_4$ as the mobile phase. After the optimisation, the method was applied for the quantification of lactic acid enantiomers in several red and white wine samples, collected from two private wineries from Romania. The concentration of L-lactic acid ranged between 0.79 and 1.24 g/l, while D-lactic acid was between 0.17 and 0.29 g/l.

Key words**:** *lactic acids, enantiomers separation, wine sample, malolactic fermentation*

INTRODUCTION

There are many studies focused on investigating the positive action of organic acids on the human body [15, 8, 14]. It was proven that some organic acids have high antioxidant power (ascorbic acid) [8], antibacterial activity (benzoic and salicylic acids), anti-inflammatory (hydroxycinnamic acids), antimutagenic, anticarcinogenic or anti-inflammatory properties (gallic acid) [15], protective effects on the myocardium (citric acid, malic acid) [17], enhance the iron absorption (succinic acid, acetic acid, citric acid, lactic acid, malic acid, glutamic acid) [1].

The organic acids content of wine is of great interest since these compounds influence the wine's sensory properties, like flavour, taste, colour or aroma [12]. Tartaric and malic acids are the main organic acids found in wine and they originate from the grapes, while other acids like lactic, succinic or acetic, are found in lower concentrations and are formed during the alcoholic and malolactic fermentation. The most important acids present in wine are tartaric acid, malic acid, and lactic acid [2]. Lactic acid is a highly desirable component in some wines because it leads to wine softening, fruity and vegetative aromas. Therefore, many wines undergo a process of malolactic fermentation, where malic acid is decarboxylated into lactic acid, a process commonly found in red wines and some white wines [9, 18].

Lactic acid is present in two enantiomeric forms, which are differently metabolized in the human body. Thus, L(+)-lactic acid is present as a metabolic intermediate, while the isomer D(–)-lactic acid is excreted from the body [10]. Because of their different effects, the two enantiomers have different practical applications, such as: the chemical production of some plasticizers, adhesives or household cleaners for D(–) isomer, while L(+)-lactic acid is used in skin care products, as preservative in food processing, antioxidant, flavouring agent (E270), pH regulator, etc. [10,19]. The predominance of L-lactic acid in

wine is generally associated with malolactic fermentation [9], while the production of D-lactic acid can indicate wine spoilage.

Different methods like spectrophotometric, enzymatic, nonenzymatic, chromatographic and electrophoretic have been developed to quantify the organic acids in wines [12, 3, 13]. A detailed presentation of these techniques can be found in [14]. Considering the high efficiency for enantioselective separation, high-performance liquid chromatography with UV or mass spectrometry detection, using chiral stationary phases, is the most widely used method for lactic acid enantiomers separation [10].

In the present study, a sensitive analytical method by HPLC was optimised for the simultaneous determination of the two enantiomers of lactic acid in wine samples. The method was used to quantify L and D-lactic acid in several wine samples.

MATERIALS AND METHODS

Chemicals and standard solutions

The L-lactic acid standard (100 mg) was purchased from Supelco, while the D-lactic acid standard (250 mg) was purchased from Alfa Aesar.

Mobile phase (aqueous solution of $CuSO_4$, 5 mM) was prepared from $CuSO_4$ anhydrous (10 g), without traces of metals (99.99%) purchased from Sigma-Aldrich.

All the dilutions were made with HPLC pure water (Promochem).

Wine sampling and processing

For this study, wine samples from two private vineyards (Pietroasele – Buzău County and Ștefănești – Argeș County) located in an important Romanian wine region, namely Muntenia region, were analysed (Map 1).

Four samples were tested, consisting of two red wines and two white wines. The wine samples were taken from freshly opened bottles and filtered through Nylon membrane syringe filters of 0.2 µm porosity.

In order to check if the dilution of the wine sample is required before the HPLC analysis, the general physico-chemical parameters (pH, redox potential, electrical conductivity – EC and salinity) were measured by using a multiparameter (WTW 520i).

Following these measurements, it was decided to dilute the wine sample with pure water in a ratio of 1:5 (*v:v*) before HPLC analysis.

Map 1. Location of the two vineyards where the analysed wines come from
Source: Modified map after [20, 21].

HPLC analysis

The HPLC system (Agilent 1200) consisted of a binary pump, a degassing device, a 20 µl injection loop, and a UV-Vis Diode Array Detector. The chromatographic separation was performed on an Astec CLC-D Chiral HPLC Column (Sigma Aldrich), 15 × 0.46 cm ID, 5 µm particle size, 100 Å for pore diameter. According to supplier instructions, the mobile phase consisted of 5mM $CuSO_4$ aqueous solution (pH = 4.2), in isocratic elution at a flow rate of 1 ml/min. The injection volume was 20 µl. The analysis was performed at room temperature.

To optimize the HPLC method, several specific parameters (column separation efficiency, linearity, sensitivity, precision and accuracy) were measured, as presented below.

To establish the optimal conditions for liquid chromatographic separation of the two enantiomers of lactic acid, the *separation efficiency of the column* was tested by calculating the *resolution factor (R_S)* using the formulae [5,16]:

$$R_S = 2 \cdot \frac{t_{R_2} - t_{R_1}}{w_1 + w_2} \quad (1)$$

where:
R_s – resolution factor,
t_{R1} –the time between injection and detection of the first analyte (L-lactic acid),
t_{R2} – time between injection and detection of the second analyte (D-lactic acid), and
w – peak width.
For a satisfactory separation of peaks with different heights, the value RS ≈ 1 is accepted for the resolution factor [5,16].
The *linearity* was tested by an external standard calibration method, by analysing a series of four standard solutions (0.15, 0.6, 0.9, 1.5 g/l) of L-lactic acid and D-lactic acid. The peak areas were plotted versus the concentration to get the regression equation and coefficient of determination (R^2) for the calibration curves. The analyses were performed at five different wavelengths (210, 230, 249, 254 and 259 nm).
Method *sensitivity* was evaluated by calculating the limit of detection (LOD) and limit of quantification (LOQ), according to International Conference on Harmonisation (ICH) guidelines, using the formulae [7, 4]:

$$LOD = 3.3 \cdot \frac{\sigma}{S} \quad (2)$$

$$LOQ = 10 \cdot \frac{\sigma}{S} \quad (3)$$

where:
σ – the standard deviation of response (standard deviation of blank response) and
s – the slope of the calibration curve.
The *precision* of the HPLC method was determined by repeatability (intra-day) and intermediate precision (inter-day) [7,11]. The repeatability tests were performed by injecting standard solutions (0.6 and 1.5 g/l) of L and D-lactic acid on the same day and by calculating the relative standard deviation (RSD). The intermediate precision was evaluated by analysing standard solutions (0.6 and 1.5 g/l) on different days, but in identical analytical conditions.
The *accuracy* of the HPLC method was evaluated based on the recovery study. Samples of white wine from Ștefănești, diluted with ultrapure water to 1:1 ratio, were spiked with known amounts of standard solutions of L and D-lactic acid. The recovery was calculated based on the analyte concentrations before and after spiking.

RESULTS AND DISCUSSIONS

HPLC method optimisation

Following the tests, it was observed that the retention of enantiomers L and D on the used column is strong, the *resolution factor* R_s having the value of 1.108, satisfying the optimal conditions for chromatographic separation (RS ≈ 1, for peaks with different heights) [5,16].
The *linearity* data, including the slope, intercept and coefficient of determination, are presented in Table 1. A coefficient of determination (R^2) higher than 0.995 indicates a good linearity for the HPLC method [11]. In the present study, the method proved to be linear, in the range of 0.15 – 1.5 g/l. The best linearity, for both L and D–lactic acid was registered at 254 nm (R^2 = 0.9998 for L–lactic acid, R^2 = 0.9999 for D–lactic acid). Consequently, all the analyses were performed at 254 nm.

Table 1. Linearity regression data depending on wavelength

Analyte	Concen-tration range (g/l)	Wavelength (nm)	Slope	Intercept	R^2
L-lactic acid	0.15 – 1.50	210	774.77	201.88	0.9424
		230	12669	585.3	0.9950
		249	11289	1134	0.9976
		254	11077	99.96	0.9998
		259	5251.5	1475.1	0.932
D-lactic acid		210	767.95	359.6	0.9558
		230	12983	301.43	0.9978
		249	10927	487.77	0.9906
		254	10732	22.86	0.9999
		259	5464.2	976.92	0.9782

Source: Own results.

The HPLC method proved to be *sensitive* to the quantification of L and D-lactic acid. The LOD was 1.17 µg/l for L-lactic acid and 1.21 µg/l for D-lactic acid, while the LOQ was 3.55 µg/l for L-lactic acid and 3.67 µg/l for D-lactic acid. The values for LOD and LOQ were similar to those mentioned in the literature [11].
The results for the repeatability and intermediate *precision* are shown in Table 2. The RSD for repeatability (intra-day) ranged between 0.92 and 1.28%, while the RSD for

intermediate precision (inter-day) was between 1.38 and 1.53%. The results showed that the RSD was < 2%, for both L and D–lactic acid, indicating that the HPLC method is precise [11].

Table 2. Method precision

Analyte	Concentration of standard solution (g/l)	Intra-day		Inter-day	
		Measured concen-tration* (g/l)	RSD** (%)	Measured concen-tration* (g/l)	RSD** (%)
L-lactic acid	0.6	0.56	1.28	0.55	1.53
	1.5	1.47	0.92	1.48	1.38
D-lactic acid	0.6	0.57	1.22	0.56	1.48
	1.5	1.48	1.19	1.49	1.52

*average of three measurements,**RSD – Relative Standard Deviation
Source: Own results.

The method *accuracy* was evaluated by recovery tests for the spiked wine samples (wine samples from Ștefănești). All the analyses were performed in triplicates and the average level was calculated. The results indicated high recovery values: 99.89% for L-lactic acid and 102.2% for D-lactic (Table 3). The values were within the acceptable limits (90 – 110%) and were similar to those reported in other studies [11].

Table 3. Method accuracy – recovery test

Analyte	Concentration (g/l)			Recovery (%)
	wine sample	wine sample_spiked		
		expected	observed	
L-lactic acid	0.44	0.97	0.96	98.97
D-lactic acid	0.16	0.72	0.74	102.78

Source: Own results.

Application of the proposed method on real wine samples

The analysed wine samples had a pH between 3.4 and 4.2 (Fig. 1). The tested wines had a pH within the usual range for wines, which is between 3 and 4, with white wines being generally more acidic than red ones. The pH level is an important parameter, which can impact the wine's colour or its taste and smell.
The electrical conductivity of the wine samples ranged between 1,650 and 2,090 µS/cm (Fig. 2). Considering the relatively high conductivity, it was decided to dilute the wine sample with pure water, in a ratio of 1:5 (*v:v*) before HPLC analysis.

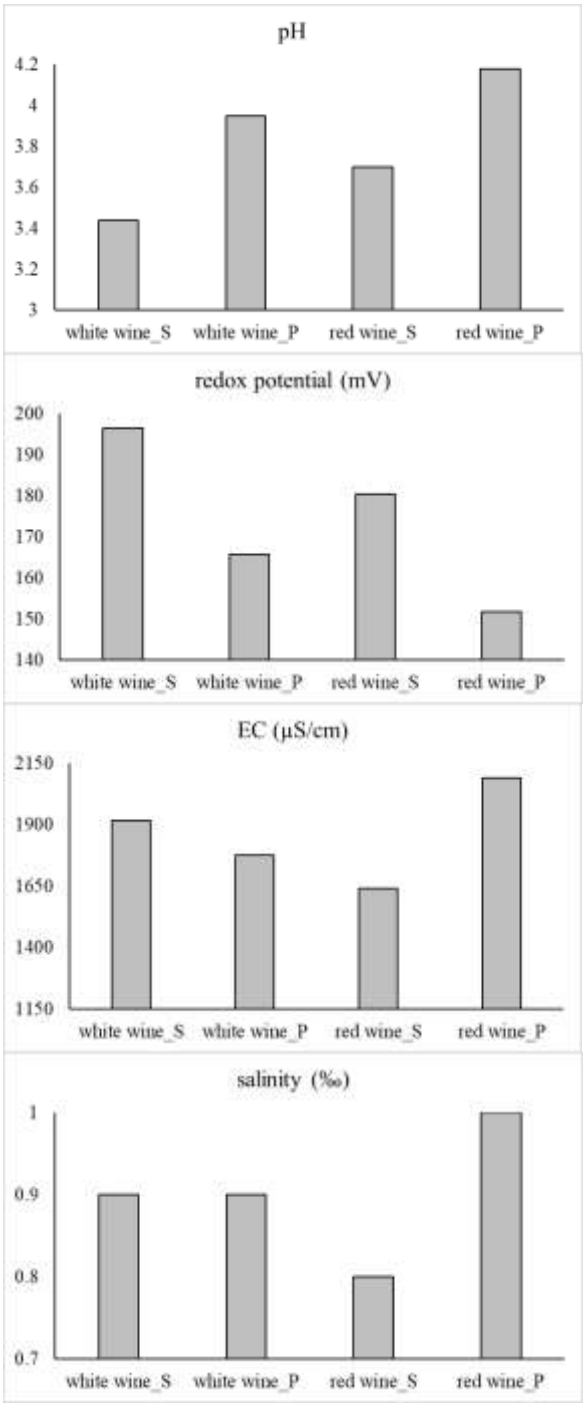

Fig. 1. Physico-chemical parameters of the analysed wine samples
Source: Own results.

Once the chromatographic conditions were established, the proposed method was applied to determine the concentration of L-lactic acid and D-lactic acid in two white wine samples and two red wine samples, from two private wineries from Romania. The amounts of the two enantiomers are presented in Fig. 2.

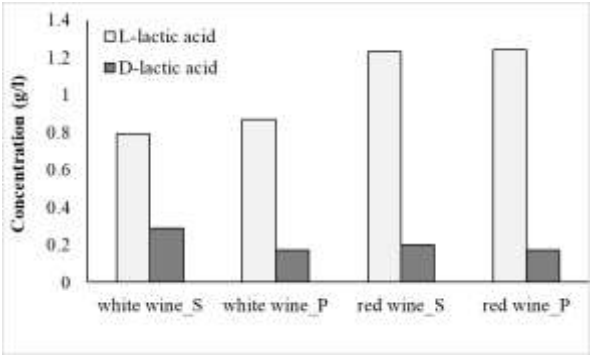

Fig. 2. Concentration of L and D-lactic acid in wine samples from Săndulești (S) and Pietroasele (P) wineries
Source: Own results.

The results showed that the concentration of L-lactic acid (0.79 – 1.24 mg/ml) was higher than D-lactic acid (0.17 – 0.29 mg/ml). The level of L-lactic was higher in red wine than in white wines, while the D-lactic acid was higher in white wines. Considering that the concentration of L-lactic acid is four–five times higher than the level of D-lactic acid, it results that the malolactic fermentation started in the analysed wine samples.

The results obtained in the present study are similar to those reported in other studies. Han et al. [6] investigated the content of several organic acids in 12 wine samples from different regions and reported the following concentrations for L-lactic acid: 0.05 – 2.21 g/l (France region), 0.01 – 0.08 g/l (Germany), 0.01 – 0.07 g/l (Italy), 0.17 g/l (Spain) and 0.39 – 1.57 g/l (USA). According to the same study, the concentration of D-lactic acid ranges between 0.12 – 0.14 g/l (France region), 0.09 – 0.10 g/l (Germany), 0.13 – 0.16 g/l (Italy), 0.10 g/l (New Zealand), 0.12 g/l (Spain) and 0.13 – 0.26 g/l (USA).

CONCLUSIONS

The results of the present study showed that the proposed HPLC method can be successfully used for the quantification of L and D enantiomers of lactic acid in wine samples. The new stationary phase showed good sensitivity and a suitable performance. An advantage of the proposed method is that the sample does not require complex treatment procedures or pre-treatment steps, like solid phase extraction or derivatisation, before HPLC analysis. Sample preparation consisted of dilution and filtration.

Based on the stereoisomer concentration results the malolactic fermentation started in the analysed wine samples.

Optimizing the analysis method of lactic acid enantiomers is of great importance considering his role on defining the wine's sensory properties, being responsible for reducing the acidity of wines and providing a smoother flavour.

ACKNOWLEDGEMENTS

The present study was funded by the research project “Multiparametric system for wine monitoring fermentation (SENS-WINE)”, project number PN-II-PT-PCCA-2013-4-0297, Contract number 146/2014.

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