Enzymatic determination of L(+) lactic and L(−) malic acids in wines by flow-injection spectrophotometry

José L.F.C. Lima[a], Teresa I.M.S. Lopes[b], António O.S.S. Rangel[b,*]

[a] *CEQUP/Departamento de Química-Física, Faculdade de Farmácia, Universidade do Porto, Rua Aníbal Cunha 164, 4050 Porto, Portugal*
[b] *Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Dr. António Bernardino de Almeida, 4200 Porto, Portugal*

Abstract

A flow-injection system for the enzymatic determination of L(+) lactic acid and L(−) malic acid in wines with spectrophotometric detection is described. The samples are dialysed in-line, and the enzymes in solution (malate dehydrogenase and lactate dehydrogenase) are injected as a train of plugs in the acceptor stream of the dialysis unit, yielding two peaks corresponding to the NADH formed for each determination. This methodology enables the determination of both acids with a single detector with a sampling rate of 20 h^{-1} (0.4–3 g l^{-1}). The results are comparable to those obtained by the reference procedure, the repeatability is better than 5% (rsd), with low enzyme consumption (1.3 μl of suspension per sample).

Keywords: Sequential flow injection; L(+) lactic acid; L(−) malic acid; Spectrophotometric enzymatic determination; Wines

1. Introduction

The determination of lactic and malic acid is frequently performed in oenological laboratories as they strongly influence the quality of the wines [1]. During malo-lactic fermentation, malic acid is converted to lactic acid and the respective contents must be monitored. Therefore, it is important to devise methodologies for measuring both acids simultaneously.

For the simultaneous analysis of organic acids, HPLC is usually used [2], while the determination of individual acids is currently performed by colorimetric or enzymatic methodologies [2,3]. The enzymatic methods are advantageous in terms of selectivity and sensitivity, but the reagents are expensive and the conventional batch analytical procedure is very time-consuming. These drawbacks can be minimized if these determinations, namely for malic and lactic acids, are run in continuous flow systems, as it was demonstrated using segmented flow manifolds [4], and more recently in flow-injection systems. The enzymatic determination of L(−) malate in wines by FIA, using soluble enzymes and spectrophotometric detection [5], and separately for the quantification of both acids [6] were reported. Regarding the simultaneous determination of L(+) lactic and L(−) malic in wines, manifolds using in-line enzyme immobilized reactors and fluorometric [7] or electrochemical detection [8] were described.

*Corresponding author. Fax: 00351 2 558 0063; e-mail: rangel@esb.ucp.pt

In this work, a flow-injection system with spectrophotometric detection was developed to make the determination of both acids in wines. A train of dissolved enzymes was injected into a buffer carrier stream, flowing to a dialysis unit to receive the wine (donor stream) components diffused across the membrane. The dialysis process allowed in-line concentration adjustment and minimized the intrinsic sample absorption. The methodology is based on the reaction [3] of L(+) lactic and L(−) malic acids with the nicotinamide adenine dinucleotide (NAD) coenzyme, catalyzed by the enzymes L(+) lactate dehydrogenase (LDH) and L(−) malate dehydrogenase (MDH), in the presence of hydrazine and at a pH of 9.5. The amount of the reduced form of the dinucleotide (NADH) produced was measured at a wavelength of 340 nm.

2. Experimental

2.1. Instrumentation

The flow system comprised two Gilson Minipuls 3 peristaltic pumps, a laboratory made commutator [9] as injection system, and Omnifit PTFE tubing (0.8 mm i.d.) as manifold conduits. Reactor R_2 (Fig. 1) was immersed in a thermostated water bath (37°C). The dialysis unit intercalated in the manifold presented a configuration similar to the one previously described [10] with a flow channel 2 mm wide and 0.5 mm deep and a linear path length of 70 mm. This unit was made of two blocks of acrylic pressed against each other by four screws. A cellulose acetate dialysis membrane with a 8000 D molecular weight cut-off value was placed between the two blocks (separating the donor and acceptor streams). The same membrane was used for more than two months with no evidence of alteration of its working characteristics.

The detector was a Hitachi 100–40 UV/Vis spectrophotometer with an Hellma 178.711-QS flow cell (10 mm path length, 30 μl optical volume) for the FIA measurements. The spectrophotometer was coupled to a Kipp and Zonen BD 111 chart recorder.

2.2. Reagents and solutions

All solutions were prepared with de-ionised water with a specific conductance <0.1 $\mu S\ cm^{-1}$, and analytical reagent-grade chemicals.

The buffer solution was prepared weekly by dissolving glycine (37.5 g), hydrazine sulphate (26 g) and EDTA (1 g) in 250 ml of NaOH 2 $mol\ l^{-1}$ solution. The pH of this solution was adjusted to 9.5 with the same NaOH solution and the volume was completed to 500 ml with water. The de-ionised water used to prepare this solution was previously boiled to avoid the formation of air bubbles inside the flow system.

The LDH/NAD solution was daily prepared by adding 0.075 g of NAD (GRAD III, 90%, Boehringer ref. 710113) to 0.50 ml of LDH suspension (specific

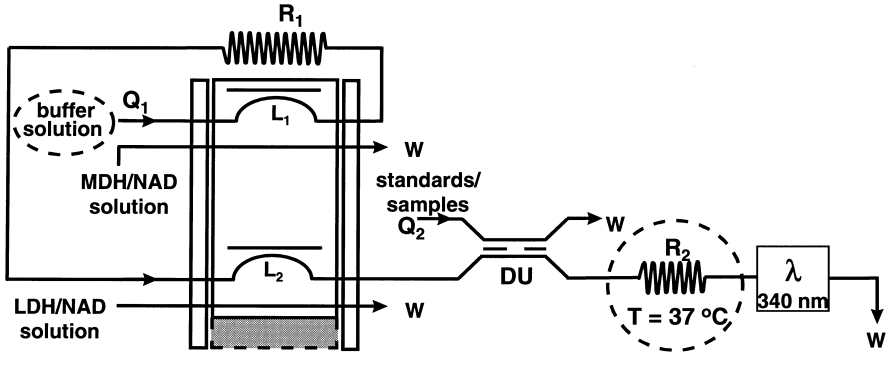

Fig. 1. FIA system for the sequential enzymatic determination of L(+) lactic and L(−) malic acids. Buffer solution (pH =9.5): 75 $g\ l^{-1}$ glycine, 52 $g\ l^{-1}$ hydrazinum sulphate and 2 $g\ l^{-1}$ EDTA; LDH/NAD solution: 7.5 $g\ l^{-1}$ NAD^+, 50 $\mu l\ ml^{-1}$ LDH suspension; MDH/NAD solution: 7.5 $g\ l^{-1}$ NAD^+, 50 $\mu l\ ml^{-1}$ MDH suspension; Loops: L_1=L_2=25 μl; Reactors length: R_1=300 cm, R_2=75 cm; Flow rates: Q_1=Q_2=1.3 $ml\ min^{-1}$; DU=dialysis unit; W=waste. The manifold components within dashed lines (buffer and reactor R_2) were kept immersed in a temperature controlled water bath (T=37°C). The shaded area is an alternative permissible position of the commutator.

activity 550 U mg^{-1}, Boehringer ref. 127876). The volume was completed to 10.0 ml with the buffer solution.

The MDH/NAD solution was prepared as the previous one, using MDH (specific activity 1200 U mg^{-1}, Boehringer ref. 127914) instead of LDH suspension.

The working standard solutions of L(+) lactic and L(−) malic acids were prepared from their respective solids. Each standard was composed by a mixture of both acids in the 0.4–3 g l^{-1} range.

2.3. Flow-injection configuration

The developed manifold is shown in Fig. 1.

In the filling position, loop L_1 was filled with the MDH/NAD solution and loop L_2 with the LDH/NAD solution. The standards, or wine samples without any pre-treatment, were pumped through Q_2 channel which is the donor stream of the dialysis unit. The analytes that diffused across the dialysis membrane were received by the buffer solution that flowed continuously through the system (Q_1), working simultaneously as carrier and acceptor stream in the dialysis process. The resulting stream passed through reactor R_2 (that was immersed in a thermostatic bath at 37°C) towards the detector. The baseline obtained in these conditions corresponded to the blank measurement, resulting from absorbing species from the wines that crossed the dialysis membrane. The dialysis unit allowed to minimize the intrinsic absorption of the samples at this wavelength, and also produced an in-line dilution to fit the solutions composition to the linear working range of the spectrophotometric measurement.

When the commutator was switched, the two enzyme/NAD solutions (L_1 and L_2) were simultaneously introduced into distinct points of the system (with the LDH plug ahead, and separated by reactor R_1) and were transported by the buffer solution towards the dialysis unit. There the train of enzyme solutions contacted with the diffused analytes, and NADH formation proceeded in reactor R_2, being the absorbance measured at the flow cell. The signal obtained presented two peaks, the first corresponding to the determination of L(+) lactic acid and the second one to L(−) malic acid. As the specific activity of the MDH suspension was higher (almost double) than the LDH suspension, the last one was chosen to be the first enzyme to be introduced into the manifold.

2.4. Reference procedure

The batch conventional procedures was carried out similarly as described in Ref. [3]. To a cuvette 0.9 ml of buffer solution, 2 ml of previously diluted (1 : 100) wine and 0.1 ml of 40 g l^{-1} NAD solution, were added. After measuring the absorbance (A_1) of this solution at 340 nm, 4 μl of MDH (or 8 μl of LDH) suspension was added and the mixture incubated at 37°C for 30 min. After cooling to room temperature, the absorbance (A_2) was read at the same wavelength. The concentration was calculated by interpolating the absorbance difference (A_2-A_1) in a calibration plot obtained with standards with concentrations ranging from 3 to 30 mg l^{-1}.

3. Results and Discussion

3.1. Optimisation of the manifold

The flow-injection system was devised to allow the simultaneous determination of L(+) lactic and L(−) malic acids, with direct introduction of the wine samples.The parameters of the FIA system were firstly selected in order to attain a sufficient separation of the two signals corresponding to each determination. The manifold was then optimised to minimise enzymes consumption and to obtain a good sensitivity and sampling rate.

For preliminary studies to select conditions for peaks separation, the volume of the enzyme loops were set to 25 μl and the length of the reactor R_2 was fixed to 75 cm. Using a flow rate of 0.9 ml min^{-1} for the buffer and standard/sample solutions, reactor R_1 was changed from 100 to 300 cm. Only for 300 cm, it was possible to guarantee that the peaks were sufficiently separated, as the peak heights obtained with the simultaneous injection of both enzymes were identical to the ones registered with the injection of just one enzyme plug (L_1 or L_2). The length of reactor R_1 was then set to 300 cm.

For these conditions, and for a concentration of 50 μl ml^{-1} for each enzyme, the concentration of NAD was varied from 1.0 to 7.5 g l^{-1}. Calibration

curves were established and the sensitivity (defined as the slope of the obtained calibration curves) increased all over this interval, but with a tendency to stabilize at the end of the interval, and so a concentration of 7.5 g l^{-1} was chosen.

Regarding enzymes concentration, there was almost a linear increase in the sensitivity as MDH or LDH concentration was augmented; a concentration of 50 μl ml^{-1} was chosen as a compromise between sensitivity and enzyme consumption.

In order to achieve a higher sampling rate, the influence of the flow rates of both the buffer (Q_1) and the standard/sample solutions (Q_2) were evaluated; in the 0.8–1.3 ml min^{-1} range (maintaining equal values for both channels), the sensitivity of the method was not significantly affected. However, the sampling rate increased from 25 to 40 determinations per hour. For flow rates higher than 1.3 ml min^{-1}, the peak height decreased and so the flow rate for both channels was set to 1.3 ml min^{-1}.

In these conditions, the absorbance was linearly related to the concentration of both acids in the concentration range from 0.4 to 3 g l^{-1}. The detection limit was determined as 0.05 g l^{-1} for L(+) lactic acid and 0.09 g l^{-1} for L(−) malic acid, calculated according to IUPAC recommendations [11]. The consumption of enzymes suspension was 1.3 μl per sample.

3.2. Application to wine samples

Port and Table wine samples, both red and white, were introduced into the FIA system without any previous treatment. The validity of the proposed procedure was checked by the analysis of twenty two wine samples by FIA and the reference procedure (Table 1).

From the linear regression obtained from the comparison of the two methods for L(+) lactic acid, the 95% confidence limits obtained [12] for 20 degrees of freedom (t-value=2.09) were -0.02 ± 0.13 g l^{-1} for the intercept and 1.00 ± 0.07 g l^{-1} for the slope; the corresponding linear regression for L(−) malic acid showed that the 95% confidence limits for 20 degrees of freedom were -0.02 ± 0.08 g l^{-1} for the intercept

Table 1
Results obtained by the developed system (FIA) and by the reference procedure (RP) and relative deviations (RD)

Sample	L(+) lactic acid			L(−) malic acid		
	FIA (g l^{-1})	RP (g l^{-1})	RD (%)	FIA (g l^{-1})	RP (g l^{-1})	RD (%)
1	0.96	0.93	+3.2	1.31	1.39	−5.8
2	1.42	1.47	−3.4	0.97	0.95	+2.1
3	2.06	2.00	+3.0	1.06	1.05	+1.0
4	1.21	1.30	−6.9	0.47	0.49	−4.1
5	1.41	1.47	−4.1	1.61	1.63	−1.2
6	0.95	1.01	−5.9	2.49	2.50	−0.4
7	2.64	2.52	+4.8	0.98	1.04	−5.8
8	2.43	2.33	+4.3	1.97	1.89	+4.2
9	2.45	2.47	+0.4	1.51	1.60	−5.6
10	1.64	1.66	−1.2	1.31	1.30	+0.8
11	1.84	1.82	+1.1	1.18	1.29	−8.5
12	1.27	1.29	−1.6	1.59	1.63	−2.5
13	2.93	2.90	+1.0	1.33	1.44	−7.6
14	1.69	1.65	+2.4	1.77	1.78	−0.6
15	1.40	1.49	−6.0	1.03	1.05	−1.9
16	0.60	0.70	−14.3	0.31	0.34	−8.8
17	2.41	2.56	−5.9	0	0	0
18	0.53	0.55	−3.6	0	0	0
19	2.40	2.67	−10.1	0.42	0.43	−2.3
20	1.02	1.16	−12.1	1.31	1.46	−10.3
21	1.40	1.29	+8.5	1.51	1.42	+6.3
22	1.04	0.96	+8.3	1.84	1.78	+3.4

and $1.00 \pm 0.06\ g\ l^{-1}$ for the slope. The values here presented demonstrate the good agreement between methods.

The relative standard deviations for five repeated injections of three wine samples were 4.9% ($0.61\ g\ l^{-1}$), 3.4% ($1.28\ g\ l^{-1}$) and 4.2% ($2.36\ g\ l^{-1}$) for lactic acid, and 3.1% ($0.99\ g\ l^{-1}$), 4.3% ($1.35\ g\ l^{-1}$) and 3.6% ($2.59\ g\ l^{-1}$) for malic acid. The sampling rate achieved was of $20\ h^{-1}$, with a low enzyme consumption (1.3 µl of suspension per sample).

4. Conclusions

The proposed methodology is a good alternative to both the reference procedure and some previously described flow methodologies, as it allows the simultaneous determination of L(+) lactic and L(−) malic acids in wines, without any previous treatment of the samples. Additionally, the detection system used is available in most laboratories of routine wine analysis, being the same used in the reference procedure.

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References

[1] M.A. Amerine, C.S. Ough, Methods for Analysis of Musts and Wines, Wiley, New York, 1980.
[2] Office International de la Vigne et du Vin (OIV), Recueil des Méthodes Internationales d'Analyse des Vins et des Moûts, OIV, Paris, 1990.
[3] J. Ribéreau-Gayon, E. Peynaud, P. Sudraud, P. Ribéreau-Gayon, Sciences et Techniques du Vin (Tome 1: Analyse et Contrôle des Vins), Bordas, Paris, 1982.
[4] J.L. Battle, R. Joubert, Y. Collon, C. Jouret, Ann. Fals. Exp. Chim. 71 (1978) 223.
[5] C. Garcia De Maria, T.M. Muñoz, A.A. Mateos, Anal. Chim. Acta 247 (1991) 61.
[6] J.L.F.C. Lima, A.O.S.S. Rangel, Am. J. Enol. Vitic. 43 (1992) 1.
[7] R. Puchades, M.A. Herrero, A. Maquieira, J. Atienza, Food Chem. 42 (1991) 167.
[8] S. Yoshioka, H. Ukeda, K. Matsumoto, Y. Osajima, Electroanalysis 4 (1992) 545.
[9] H. Bergamim F, J.X. Medeiros, B.F. Reis, E.A. Zagatto, Anal. Chim. Acta 101 (1978) 9.
[10] J.L.F.C. Lima, O.B.A.O. Neves, A.O.S.S. Rangel, in: J.J. Bimbenet, E. Dumoulin, G. Trystram, Automatic Control of Food and Biological Processes, Elsevier, Amsterdam, 1994, p. 67.
[11] International Union of Pure and Applied Chemistry (IUPAC), Anal. Chem. 48 (1976) 2294.
[12] J.C. Miller, J.N. Miller, Statistics for Analytical Chemistry, 3rd ed., Ellis Horwood, Chichester, 1993.