Enzymatic Determination of L(-)malic and L(+)lactic Acids in Wine by Flow Injection Analysis

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The enzymatic determination of L(-)malic and L(+)lactic acids in several types of wines by flow injection analysis (FIA) with spectrophotometric detection is described. This flow injection system, which incorporates a dialysis unit for adjusting the composition of the injected solutions to the requirements of the measuring system, enables determinations of these two organic acids without the need for any prior treatment of the wine samples, with a concentration interval of between 0.02 and 4 g/L, and a sampling rate of approximately 20 determinations per hour. The results obtained with this FIA method for various types of Portuguese wines are in good agreement with those of the batch method which uses the same enzymatic technique and are quite precise as they present a coefficient of variation below 2.5%.

KEY WORDS: flow injection analysis, L(-)malic acid, L(+)lactic acid, spectrophotometric enzymatic determination, wine

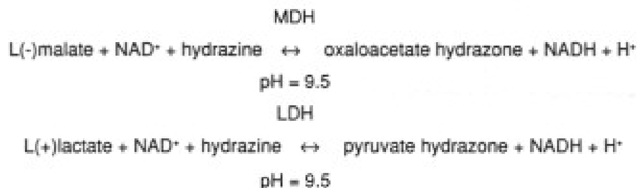
There has been a considerable increase in the use of enzymatic analytical methods over the past years, mainly because of their high selectivity and sensitivity. These advantages, however, do not always justify their routine use because of the high cost of the reagents required and their instability in solution, as well as the considerable manipulations involved.

There are definite advantages in automating the enzymatic methods to increase the sampling rate and, at the same time, cut down on the unit cost of the determinations by decreasing the amount of the necessary reagents.

This also applies for enzymatic determinations in wines, namely for L(-)malic and L(+)lactic acids for which automated segmented flow analysis (SFA) methods have already been described in several papers (2,3,8) and equipment is available on the market.

In this paper, we describe the use of flow injection analysis (FIA) (10) in the automation of these determinations as an alternative to those systems which currently are used only in large laboratories. The method has proven particularly attractive for enzymatic analysis in many fields (4), since by using simple, versatile, and inexpensive equipment, it results in good sampling rates and economizes on samples and reagents.

The FIA system presented is based on the reaction of L(-)malic and L(+)lactic acid to the nicotinamide adenine dinucleotide (NAD) coenzyme, catalyzed by the L(-)malate (MDH) or L(+)lactate (LDH) dehydrogenase enzymes at a pH of 9.5, in the presence of excess hydrazine and NAD^+, to extend the reaction in the formation of products:



A wavelength of 340 nm was used to measure the amount of the reduced form of the dinucleotide (NADH).

Materials and Methods

Instrumentation: The FIA and batch methods absorbance measurements were carried out on a Hitachi Model 100-40 UV/VIS spectrophotometer equipped with a Helma ref. 178.711-QS flow cell (30 µL of optical volume), connected to a Metrohm Model E 586 Labograph recorder. Normal parallel-piped quartz cells with a 1-cm flow path were used for the batch method.

The solutions were de-gassed with a Heat Systems-Ultrasonics, Inc. ultrasonic bath.

Flow injection system: The flow injection manifold shown in Figure 1 was used for the determinations of the two organic acids.

A Gilson Model Minipuls 2 peristaltic pump and Gilson pumping tubes were used for propelling the solutions.

The samples and standards were introduced into the FIA manifold with a Rheodyne Type 50 injection valve.

Omnifit 0.5-mm i.d. PTFE flow tubes, Gilson end-fittings and connectors, and a homemade (1) perspex confluence point linked the components of the manifold. The tube (L_3) in which the species to be measured is formed was tightly helically coiled (spiral diameter, 2 mm) and immersed in a thermostatic bath (GFL Model Thermed 5001) to maintain the temperature at 37°C.

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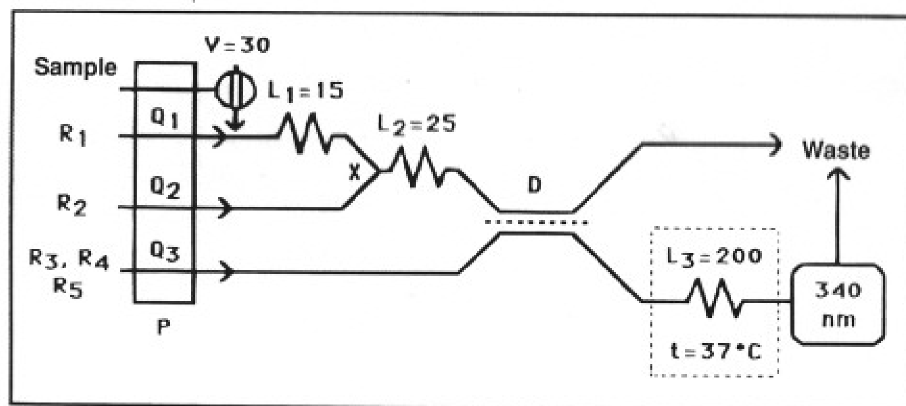

Fig. 1. Flow injection manifold used in the enzymatic determination of L(-)malic and L(+)lactic acid in wines: P, peristaltic pump; Q_i flow-rates (mL/min): $Q_1 = 0.21$, $Q_2 = 0.40$, $Q_3 = 0.43$; L_i, tube length (cm); V, injection volume (µL); *X*, confluence point; D, flow-through dialyzer unit; R_1, water; R_2, buffer solution; R_3, reagent for the malic acid determination; R_4, reagent for the lactic acid determination; R_5, reagent for blank measurements. The broken lines indicate the thermostatically controlled part of the manifold.

The Tecator flow-through dialysis unit (ref. 5000-0395) was similar to that described in (11). This had a 2.0-mm wide, 0.5-mm deep, and 7.2-cm long semi-tubular groove between the entrance and the exit slit. TDF membranes used for the segmented flow analyzers dedicated to wine analysis were used. These membranes were conditioned in water for one hour before the first usage in order to ensure good reproducibility. These membranes continued in good operating conditions during the two weeks in which they were regularly tested throughout each working day.

Reagents and solutions: Doubly deionized water (specific conductivity < 0.1 µS/cm) and analytical reagent-grade chemicals were used for the preparation of the solutions.

The L(-)malic and L(+)lactic acids standard solutions were prepared daily from their respective solids.

The buffer solution (R_2, Fig. 1) was prepared from 75 g of glycine, 52 g of hydrazinium sulfate, 2 g of $Na_2EDTA \cdot 2H_2O$, 500 mL of 0.2 *M* NaOH; pH of the solution was adjusted to 9.5 with 0.2 *M* NaOH, and diluted to 1 L with water. This solution remained stable for at least one week at 4°C. The buffer solution was filtered and degassed prior to use in preparing the solutions described below.

The reagent solution for the malic acid determinations (R_3, Fig. 1) was prepared from 34 mL of the buffer solution (R_2), 64 mg of NAD (Boehringer ref. 710113) and 32 µL of MDH suspension (specific activity 1200 U/mg, Boehringer ref. 127914), and was topped up to 50 mL.

The reagent solution for the lactic acid determinations (R_4, Fig. 1) was similarly prepared, but the MDH was substituted by 64 µL of LDH suspension (specific activity 550 U/mg, Boehringer ref. 127876).

The R_3 and R_4 reagent solutions were stable over a working day; the analytical signal corresponding to the injection of standard solutions of the two acids which were periodically injected into the manifold did not change during that period.

In some cases the wines under analysis produced an intrinsic signal at the measuring wavelength which affected the value of the final result; therefore, we performed a blank measurement to compensate for the absorption of radiation at this wavelength. This was done by substituting the reagent solution (either R_3 or R_4) in channel Q_3 with a solution prepared the same way as the reagent solutions but with no enzyme (R_5, Fig. 1).

Results and Discussion

FIA manifold configuration: The same set-up was used for the determination of both organic acids, since the requirements for the sample preparation inside the manifold prior to measuring were similar for each.

The manifold was designed to produce the reactions which produce the species to be measured and to dilute the injected solutions within the set-up so that their compositions could be adjusted to the needs of the detection system. A manifold (Fig. 1) was used which incorporated a point of confluence (*X*) and a flow-through dialysis unit (D). The flow-through dialysis unit adjusted the solution composition. As the dialysis process (continuous flow) has low efficiency, it becomes a simple and effective method of obtaining high dilutions, and it substantially minimizes the intrinsic absorption of the samples. The choice for the dialysis unit over others, *e.g.*, stream splitting systems (6,7), is justified by the greater simplicity of the systems which incorporate dialysis membranes.

A point of confluence (*X*), where the buffer solution (R_2) was added through channel Q_2, was incorporated to adjust the pH of the samples and standard solutions injected into the water carrier stream (Q_1). The addition of a buffer solution at the confluence point, rather than using it as a carrier stream, ensured that there were no pH gradients along the sample plug. Moreover, the dilution which takes place at the point of confluence minimizes the build-up of residue in the flow-through dialysis donor channel, thus increasing the lifetime of the membrane and avoiding a decrease in sensitivity of the determinations (reduced permeability) over time.

The reagent solution (R_3 or R_4, for malic or lactic acid determinations, respectively) was introduced into the acceptor stream (Q_3) to produce the species (NADH) along the reactor (L_3), which links the dialysis system to the spectrophotometer, set at a 340 nm wavelength.

Optimization of the manifold: After a previous assessment during which we obtained approximate values for each parameter, we optimized the manifold

by the univariate method: *i.e.*, by varying each parameter independently and setting the remainder. This procedure was aimed at adjusting the conditions which would be used for each of the determinations in terms of working range, sensitivity, sampling rate, and reagent consumption.

L(-)malic acid determination: The flow rates for Q_1 and Q_2 were set at 0.21 and 0.40 mL/min, respectively. These values were chosen so that the flow rate in the donor stream, after passing the point of confluence (*X*), would not differ significantly from that of the acceptor stream (Q_3). The Q_1/Q_2 flow ratio ensured a *ca* seven-fold dilution of the sample (30 µL) before it entered the dialysis unit. As the buffer solution (R_2), which flows through Q_2, is diluted 1.5 times at the point of confluence, its concentration in each component of the buffer was approximately 1.5 times greater than in those used in the reagent (R_3). This ensured that the glycine, hydrazinium sulfate, and EDTA concentration was the same in both the donor and acceptor streams.

The Q_1 and Q_2 flow rates remained set in all the experiments, because lower values than those set produced a pulsed flow (oscillations in the flow rate) in channel Q_1. This resulted from the peristaltic pump operating at greatly reduced rotations, and higher values resulted in a significant decrease of the analytical signal; the efficiency of the dialysis depends greatly on the duration of the contact of the sample plug with the membrane.

A flow rate of 0.43 mL/min was used for the acceptor stream. Lower values increased the analytical signal but produced an excessive broadening of the peak and a marked decrease in reproducibility. This was possibly due to the different flow rates in the donor and acceptor streams. With higher values, there was a marked decrease in sensitivity; *e.g.*, the absorption of a 4 g/L solution dropped 47% when the flow rate was increased from 0.43 to 0.54 mL/min.

The 15-cm long tube L_1 created a slight dispersion of the sample in the carrier stream (water) before it reached the point of confluence (*X*), thereby minimizing the influence of minor alterations in the Q_1/Q_2 flow ratio on the reproducibility of the analytical signal due to the pulsations of the peristaltic pumps. Because the buffer solution is added at the point of confluence through Q_2, the tube (25 cm) was necessary for mixing both solutions to guarantee good reproducibility during dialysis.

A helically coiled 200-cm tube (L_3) was used as a reactor to minimize the dispersion of the sample plug and, at the same time, create a sufficient residence time (1.5 min) for the formation of the species to be measured. A 100-cm tube was tried as an alternative, but this resulted in a marked decrease in peak height values (*e.g.*, the absorption of a 4 g/L solution dropped 21% when the tube length was decreased from 200 to 100 cm), and the improvement in the sampling rate was not significant. With longer reactors, there was a marked broadening of the analytical signal and thus a significant decrease in the sampling rate.

A 30-µL injection volume was chosen as a compromise between sensitivity and the sampling rate. Lower injection volumes significantly decreased the reproducibility of the signals, possibly due to the lack of reproducibility during dialysis.

The compositions of the solutions were varied for each compound.

By varying the concentration of the buffer in reagent R_3 (by varying the volume of R_2 as described in **Materials and Methods**), sensitivity increased up to the chosen value (34 mL of R_2 buffer/50 mL solution) and then stabilized.

Sensitivity always increased when the enzyme was more concentrated. The value of 34 µL of enzyme suspension/50 mL was chosen as a compromise between sensitivity and reagent consumption.

Although an increased concentration of NAD also increased sensitivity, we noted a marked base-line drift at concentrations over 64 mg/50 mL.

Under these conditions, the optimized system presented a calibration curve [absorbance (A) *vs.* concentration (c) expressed in g/L] whose values were adjusted according to the following equation:

$$A = x\,c^2 + y\,c + z$$

where, up to 4 g/L: x = -0.0038; y = 0.061; z = 0.001. This relationship remained unchanged throughout one day's work, so there was no need to perform periodic calibrations.

The detection limit was 0.02 g/L, calculated as corresponding to three times the standard deviation of the background noise (5).

L(+)lactic acid determination: Using the same manifold and aforementioned respective parameters as for the determination of L(-)malic acid, we studied the composition of reagent solution R_4.

The same concentration of the buffer was used in the reagent solution, given that an increase in the former did not affect the sensitivity of the method.

An enzyme concentration corresponding to 64 µL of enzyme suspension/50mL of solution was chosen as a compromise between sensitivity and the cost of the determinations. This value is double that used in the determination of malic acid and can be explained by the fact that the specific activity of the enzymatic solution was less in the case of lactic acid (550 for LDH *vs.* 1100 U/mg for MDH).

The NAD concentration of 64 mg/50 mL was chosen for the same reasons as for L(-)malic acid. There was a considerable decrease in the analytical signal at lower values; *e.g.*, for a 4 g/L solution in L(+)lactic acid, the analytical signal decreased 37% with a 32 mg/50 mL concentration of NAD.

Under these conditions, the optimized system presented a calibration curve whose values were adjusted according to the following equation:

$$A = x\,c^2 + y\,c + z$$

Table 1. Results obtained in the determination of L(-)malic and L(+)lactic acids in 15 wine samples by FIA and by the conventional batch method (CM).

L(-)malic acid			L(+)lactic acid		
FIA (g/L)	CM (g/L)	Relative deviation (%)	FIA (g/L)	CM (g/L)	Relative deviation (%)
0.77	0.78	-1.3	0.63	0.64	-0.16
2.40	2.40	0.00	0.51	0.50	2.0
1.42	1.54	-7.79	0.68	0.64	6.2
2.60	2.57	1.17	0.11	0.12	-8.3
0.55	0.54	1.9	0.93	0.86	8.1
2.30	2.37	-2.95	0.41	0.44	-6.8
1.80	1.92	-6.25	3.25	3.32	-2.11
1.20	1.10	9.09	1.79	1.72	4.07
0.15	0.16	-6.2	2.16	2.03	6.40
1.20	1.21	-0.026	1.69	1.62	4.32
2.05	2.05	0.00	0.78	0.80	-2.5
1.02	1.07	-4.67	1.16	1.03	12.6
1.55	1.56	-0.641	0.62	0.66	-6.1
1.75	1.76	-0.568	0.40	0.42	-4.8
1.40	1.37	2.19	0.58	0.60	-3.3

where up to 4 g/L: x = -0.0052; y = 0.072; z = 0.002. This determination also did not require a periodic recalibration of the system.

The detection limit, calculated as described for the L(-)malic acid determination, was 0.02 g/L.

Application to wine samples: FIA (C_f) and conventional batch method tests using the same enzymatic methods (C_b) (9) were used to assess the quality of the results obtained (Table 1) in the determination of the L(-)malic and L(+)lactic acids in 15 samples of various types of Portuguese wines (Ports, red and white table wines).

In the FIA determinations, the samples were injected into the system without any prior treatment. A blank measurement was performed using the R_5 (without enzyme) solution in the acceptor stream (Q_3). After subtracting the absorbance value corresponding to the blank measurement, the concentration of the sample was obtained on the basis of the corresponding calibration curve. Recorder outputs obtained in the determination of L(-)malic and L(+)lactic acids in wine are presented in Figure 2 and Figure 3, respectively.

The equation $C_f = C° + S\ C_b$ was established. Thus, for L(-)malic acid determinations, C° = 0.005, S = 0.986, and the correlation coefficient = 0.997. For L(+)lactic acid determinations, C° = 0.009, S = 1.01, and the correlation coefficient = 0.997.

These results demonstrate good agreement between the FIA and the conventional batch methods.

In assessing the reproducibility of the FIA proce-

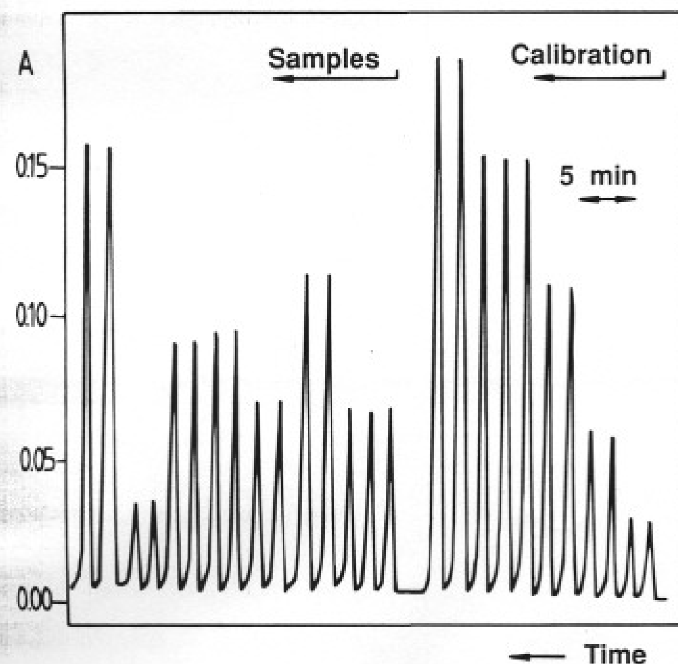

Fig. 2. Recorder output obtained in the determination of L(-)malic acid in wine, corresponding to the injection of a set of standards (with concentrations from 0.500 to 4.00 g/L) and samples.

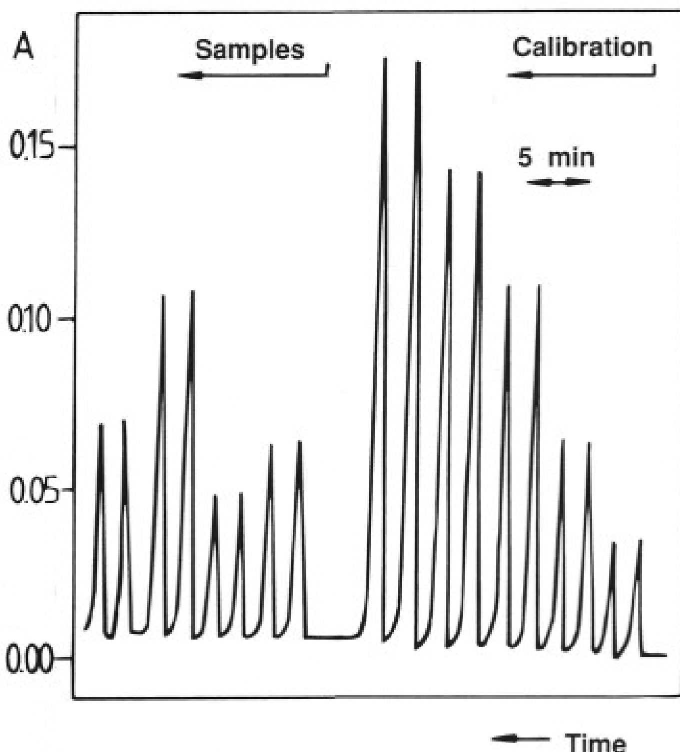

Fig. 3. Recorder output obtained in the determination of L(+)lactic acid in wine, corresponding to the injection of a set of standards (with concentrations from 0.500 to 4.00 g/L) and samples.

dure, the coefficient of variation was calculated on the basis of 10 consecutive determinations of two samples (concentrations given in brackets), corresponding to the analytical signal less that of the blank measurement. Thus for L(-)malic acid, we obtained values of 2.4 % [1.39 g/L] and 2.2 % [2.95 g/L]. For L(+)lactic acid, we obtained values of 2.2% [1.94 g/L] and 1.5% [0.84 g/L], for which the latter corresponded to seven determinations.

Conclusions

The method presented herein is a good alternative to conventional enzymatic methods used in wine analysis in which the samples are previously prepared and later measured; this method performs more determinations in the same span of time (estimated at approximately 5 five samples/hour for the batch method (3)). Furthermore, the FIA method presents lower enzyme consumption (*ca* 24-fold) per determination than the conventional method.

There also are advantages regarding the segmented flow process, as it has the same sampling rate as the method referred to in (2). It uses approximately 1.5 times less enzyme suspension, and the set-up is simpler and less expensive. Additionally it can take advantage of the fact that in FIA the analytical signal shows up 1.5 minutes after the injection of the sample, (by coupling it to a reactor where a fermentation occurs) thus enabling one to determine its evolution in close to the real time.

Should there be a malfunction of the system, the loss of reagents in the FIA system described here is much less than in SFA systems. This is due to the different foundation of the two methods. The SFA needs more solution circulating throughout the system, which would have to be totally discarded in the case of a malfunction; the amount of solution used in the FIA system is significantly less.

Finally, we note that determinations of both organic acids can be performed in the same manifold, it only being necessary to substitute the reagent solution when one changes from one determination to the other.

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