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Simultaneous Enzymatic Determination of L(−) Malic Acid and L(+) Lactic Acid in Wine by Flow Injection Analysis

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ABSTRACT

A simultaneous determination of two organic acid species in wine by a flow injection analysis method using enzyme-immobilized open tubular reactors is described. The design of the optimal geometry of the FIA system is especially important to avoid the matrix fluorescence. Reproducible results were obtained in the determination of L(−) *malic acid and* L(+) *lactic acid using* L-*MDH and* L-*LDH, respectively. The assay reactions utilize the fluorimetric determination of a common product, NADH. The sample was dialyzed on line and injected into the FIA-system. The system responds linearly (log–log calibration graphs) to the injected sample (80 µl) in concentrations ranging from 0·1 mM to 5 mM and 0·05 mM to 3 mM for malic and lactic acid, respectively. The maximum sample throughput is 15 h^{-1}. The results obtained with both proposed and HPLC methods are in good agreement. The* L-*MDH and* L-*LDH reactors were stable for six months when used daily at optimal pH.*

INTRODUCTION

The quantity and nature of organic acids are determinative to establish the quality of a wine, especially in aged red wines. The most important ones are tartaric, malic, lactic and succinic acids (Usseglio-Tomasset, 1980; Silver, 1981). Particularly, malic and lactic acid are the most frequently monitored parameters in oenological laboratories because they dramatically change

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during the malolactic fermentation and are of fundamental importance in the quality of taste (Ribereau-Gayon, 1980).

The predominant methods recently used for the simultaneous determination of organic acids are gas-chromatography (Martin *et al.*, 1971; Gump *et al.*, 1985) and high performance liquid chromatography (HPLC) (Polo *et al.*, 1986; Silva & Colagrande, 1987). Analysis times are long and detection limits are high. Furthermore, extensive sample preparation and expensive instrumentation are generally required (DeSmedt *et al.*, 1981; Marcy & Carroll, 1982).

For the specific determination of a particular organic acid a great number of enzymatic as well as colorimetric methods are available (Ough, 1988). The most common methods used in oenological studies employ enzymes to catalyze specific reactions (Amerine & Ough, 1980; Huang & Dai, 1985). Particularly, for the determination of malic and lactic acid, many examples are described in the literature (Poux, 1969; Bandion & Valenta, 1977; Batlle *et al.*, 1978; Lonvaud-Funel *et al.*, 1980; McCloskey, 1980). Conventional enzymatic determinations involve addition of the enzyme to the sample and, after an incubation period, measurement of the concentration of a substrate or product (Mollering, 1974; Noll, 1974). This has several drawbacks. Once dissolved, the enzyme often has a limited lifetime, and is not retrievable from solution, which leads to the use of quite large amounts of frequently expensive enzymes. This also limits their use in industrial laboratory control and, in general, for the analysis of large numbers of samples. These problems have been overcome by the use of immobilized enzymes. Immobilization usually stabilizes the enzyme and thus increases its lifetime, often dramatically (Ho, 1988).

Immobilized enzyme-based analyses have successfully been applied to several compounds (Mascini & Mazzei, 1987; Ohyabu *et al.*, 1987; Puichades *et al.*, 1989) and considerable attention has been given to their use in flow injection systems (Ruz *et al.*, 1988; Hansen, 1989; Tzouwara-Karayanni & Crouch, 1990). The incorporation of the selectivity of the enzyme together with the simplicity, versatility, good precision, high sampling rate, low cost, and automation of flow injection analysis (FIA) provide a useful system for a wide range of applications, particularly when the analysis of a great number of samples is required (Valcárcel & Luque de Castro, 1988). Another favourable feature of FIA is that the flow configuration can be freely constructed by analysts to suit their purposes (Morishita *et al.*, 1986). Several on-line processes such as dialysis can also be easily adapted to FIA, and the system can be interfaced with virtually any detector (Luque de Castro, 1989; Puchades *et al.*, 1990). Dialysis often overcomes the interfering effect of coloured samples.

FIA as a general analytical method and its multiple applications in

practice using immobilized enzymes are well documented in the literature; however, its applications in the oenological field have been scarce (Lázaro *et al.*, 1987).

Several enzymatic methods have been reported for the determination of lactic acid (Kanbe *et al.*, 1977; Yao *et al.*, 1982; Yao & Wasa, 1985; Petersson, 1988) and malic acid (Pinto Corraliza *et al.*, 1981; *Almuiabed & Townshend*, 1989) in flow injection systems. Such methods are available to determine these organic acids by quantifying the NADH formed when the substrate is oxidized by L-MDH and L-LDH in the presence of NAD^+ according to the following reaction:

$$\text{substrate} + NAD^+ + H_2O \overset{\text{enzyme}}{\rightleftharpoons} \text{oxaloacetate} + NADH + H^+$$

This procedure is carried out at alkaline pHs for a Michaelis constant (Km) more favourable for a reaction to the right. In most procedures a trapping agent is incorporated to remove oxaloacetate and this also drives the reaction to the right (Boehringer Mannheim, 1984). Since such trapping reactions are relatively slow, their use is not recommended for flow injection analysis (Guilbault, 1984).

Other enzymatic FIA methods using amperometric (Gorton & Hedlund, 1988) and chemiluminescence (Nikolajsen *et al.*, 1988; Nielsen *et al.*, 1989) detection are also described in the literature for monitoring lactic acid fermentation variables.

In this work a new method is reported for the simultaneous enzymatic determination of malic and lactic acid in wine. L-MDH and L-LDH were immobilized in controlled pore glass beads which were packed into a single bead string reactor (Mottola, 1983) and used in an FIA manifold. The NADH generated by the enzymatic reactions is monitored in a flow-through fluorimetric detector, which provides a high sensitivity. An on-line dialysis of the sample was carried out before the injection valve and a blank reactor was used to measure the contribution of the sample interferences to the analytical signal.

MATERIALS AND METHODS

Reagents

L-Malate dehydrogenase (L-MDH, EC 1.1.1.37 from pig heart, 1645 U/mg protein) and nicotinamide adenine dinucleotide (NAD^+, free acid, grade I, 100%) were commercially available from Boehringer Mannheim (Barcelona, Spain). L-Lactate dehydrogenase (L-LDH, EC 1.1.1.27 type XI, from

rabbit muscle, 850 U/mg protein), 3-aminopropyltriethoxysilane (99%) and controlled pore glass (CPG 240–200) were obtained from Sigma Chemical Co. (St Louis, USA). The pore size and particle size were 242Å and 100–200 mesh, respectively. Glutaraldehyde (25%), malic (99·5%) and lactic acid (90%) were purchased from E. Merck (Darmstadt, Germany). All other reagents were commercially available and of analytical grade.

The carrier solution consisted of 0·05M carbonate–bicarbonate buffer, pH 9·7, containing 1·5 mM NAD^+ and 1 mM EDTA.

Stock solutions of 20 mM malic acid and lactic acid were prepared by dilution of the appropriate weighed quantity in water. Subsequent dilutions were made to obtain the desired concentrations of working standard solutions.

All solutions were prepared with distilled and deionized water. Commercially available samples of several wines were used.

Preparation of immobilized enzyme reactors

The enzymes L-MDH and L-LDH were covalently immobilized according to the procedure described by Masoom and Townshend (1984).

Each enzyme was immobilized on alkylamine glass beads with glutaraldehyde through Schiff base formation. To 3 ml of the solution of enzyme was added 0·3 g of glutaraldehyde-treated glass beads. The solution was kept at 4°C for 2·5 h, and washed with cold distilled water and cold phosphate buffer to ensure the removal of any unlinked enzyme.

Immobilization yields were calculated by measuring protein (Bradford protein assay, Bio-Rads Labs, Richmond, CA) of the clear enzyme-containing buffers before and after coupling to the CPG. 93·9% and 99·1% of the initial protein for L-MDH and L-LDH, respectively, were found to be bound.

The immobilized enzymes were packed as a wet slurry into 8-cm long glass columns with an inner diameter of 1·2 mm. The columns were capped with teflon end fittings. When not in use, they were stored at 4°C in 0·05M carbonate–bicarbonate buffer, pH 9·7. The activity of the immobilized enzymes was determined by a batch method described by Korenbrot *et al.* (1987). The specific activities per mg of immobilized L-MDH and L-LDH were 15% and 12% of that of the soluble enzyme, respectively.

Flow system and procedures

The basic configuration for the simultaneous determination of malic and lactic acid is shown in Fig. 1. The baseline is provided by the 0·05M carbonate–bicarbonate buffer pH 9·7, 1·5 mM NAD^+ and 1 mM EDTA

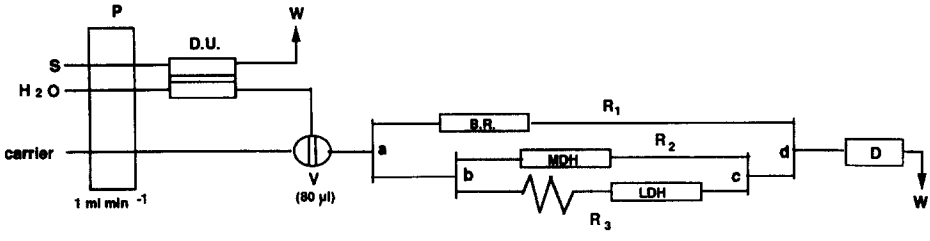


Fig. 1. Manifold for the simultaneous determination of malic and lactic acid; P, pump, S, sample; D.U., dialysis unit; V, injection valve; a-b-c-d, T-connectors; R_1, R_2 and R_3, lengths of channels; B.R., blank reactor; D, detector; W, waste.

which acts as carrier. A four-channel peristaltic pump (Model Minipuls 3, Gilson Co. France) was used to pump the carrier and water necessary for the dialysis unit at a flow rate of 1·0 ml min^{-1}. The sample is dialyzed just prior to the injection valve with a home-made plastic dialyzer (Valcárcel & Luque de Castro, 1987) using a Bran-Lubbe type C membrane. For all manifold conduits teflon tubing of 0·5 mm i.d. was used.

An 80 µl on-line dialyzed sample or standard was injected via a Rheodyne model 5041 sample injection valve into a stream containing the carrier and then flowed through the system into the detector. A T-connector was installed after the sample injection point for dividing the sample plug into two portions. One of these passes via R_1 through a blank reactor (B.R.) packed with untreated CPG for determining the matrix fluorescence. The other is divided again into two new portions which go through the R_2 and R_3 channels (both having different dimensions) and the subsequent confluence (via T-connectors) of the streams before reaching the flow cell. The L-MDH and L-LDH reactors inserted into each channel catalyze specifically the oxidation of malic and lactic acid, respectively, and produce NADH. The fluorescence intensity was measured at an excitation wavelength of 340 nm and an emission wavelength of 450 nm with a Perkin–Elmer 650-10S fluorescence spectrophotometer (Perkin–Elmer 150 Xenon power supply) equipped with a flow cell (Hellma 176·752-QS) and an HP 3392A integrator. The flow system was operated at room temperature (20°C).

The different dimensions of the three channels ($R_1 = 64$ cm, $R_2 = 95$ cm, $R_3 = 185$ cm) provided a different residence time for each one, and therefore three peaks are obtained. The first one corresponds to the fluorescence of the sample itself, the second one to malic acid and the third one to lactic acid. When standard solutions were used, only two peaks appeared. After correction of the blank value, the amounts of malic and lactic acid in wine were calculated by means of the standard curve. The peak height (arbitrary units) was used for the quantification.

Chromatographic method

Analysis of malic and lactic acid in wines was made by an automated high performance liquid chromatography (HPLC) method (Polo *et al.*, 1986) modified by Chirivella (unpublished). The method was performed by using a Varian 5000 chromatograph (Varian Instrument Group, Switzerland), a Stherisord 5 μ C-8 column (25 cm length, 0·4 cm i.d.), a Stherisord 5 μ C-8 precolumn (1 cm length, 0·4 cm i.d.) (Tracer Analytica, Barcelona, Spain), a UV/Vis detector and a Hitachi D-2500 integrator (Hitachi, Tokyo) for automatic quantitation and documentation. Three replicated measurements were carried out for each sample.

Sample treatment

At the fluorescence wavelengths used, the samples show an important emission; thus the matrix effect should be taken into account when the determinations are to be carried out. The colour of wines is one of the most important interfering effects. There are several methods of decolorizing, one of them being the use of charcoal or PVP. However, in our case this procedure did not yield good results because there was a loss of malic and lactic acid due to absorption. An HPLC method was used for malic and lactic acid determination in red wine. When the analysis was directly performed with untreated samples, the concentrations obtained were 0·73 g litre^{-1} for malic acid and 0·99 g litre^{-1} for lactic acid. On the other hand, the concentrations obtained with charcoal-decolorized samples were lower (0·48 g litre^{-1} for malic acid and 0·7 g litre^{-1} for lactic acid). Furhermore, this decolorisation procedure cannot be adapted to flow injection systems.

Another method is the use of dialysis systems; the interfering effect due to the colour was completely eliminated by dialyzing the samples with a Bran-Lubbe type C membrane. The matrix effect was smaller owing to the decolorization and the dilution caused by the dialysis, but it still existed because elements responsible for the fluorescence, such as polyphenols, dialyzed easily. This can be avoided with the proposed method, by using a blank reactor which generates a signal corresponding to the fluorescence of the sample itself. Sample treatment was unnecessary because the dialysis was carried out on line.

RESULTS AND DISCUSSION

Optimization of experimental conditions

In order to optimize the conditions for the reaction, a number of parameters such as reagent concentration, pH, carrier composition, and FIA variables

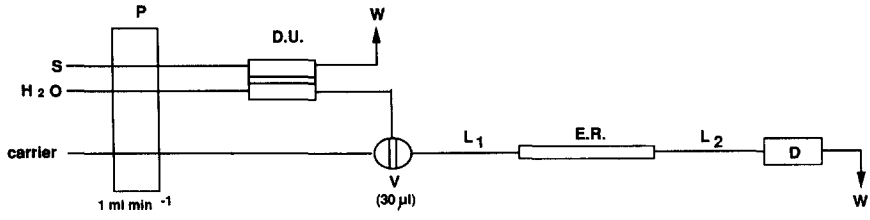


Fig. 2. Manifold for the individual determination of malic and lactic acid: P, pump; S, sample; D.U., dialysis unit; V, injection valve; E.R., enzymatic reactor; L_1, L_2, lengths of coils; D, detector; W, waste.

were investigated using the univariate method. For the optimization of the variables, the FIA system shown in Fig. 2 was used for the individual determination of L(−)malic acid and L(+)lactic acid by changing the enzymatic reactor.

Four different buffers (0·05M) at various pH values, adjusted by adding KOH or HCl in each case, were tested as carriers. The use of glycine and carbonate–bicarbonate buffers produced similar analytical signals when 1·5 mM NAD^+ and 0·5 mM malic or lactic acid standard solutions were employed. The signals obtained using phosphate buffer are lower and the borate buffer presents an inhibitor effect and cannot be used (Fig. 3). Carbonate–bicarbonate buffer was chosen for all subsequent experiments.

The pH of the carrier must be carefully chosen to ensure maximum sensitivity and stability. Figure 4 shows the effect of pH on the peak height for 0·5 mM malic acid and 0·5 mM lactic acid. The positive effect of raising the pH

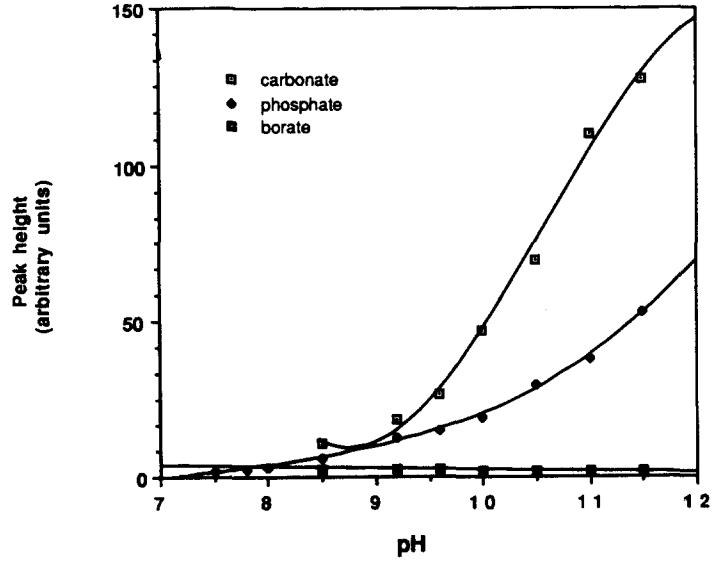


Fig. 3. Effect of different buffers on the peak height obtained for 0·5 mM malic acid at different pH values.

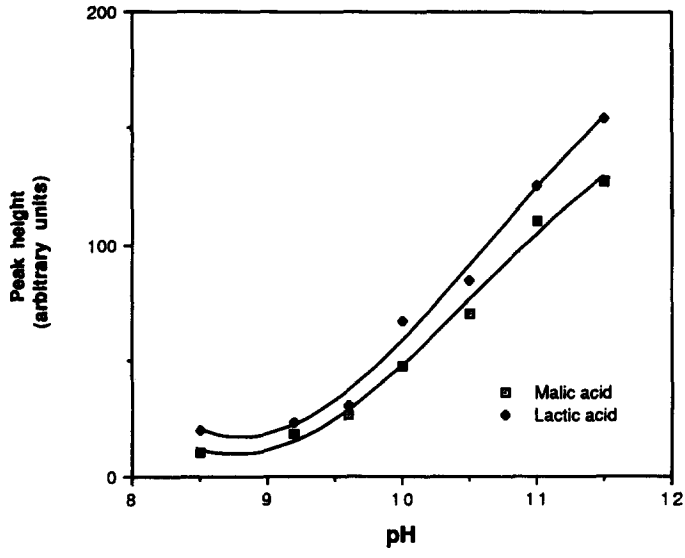


Fig. 4. Effect of pH on the response to 0·5 mM malic and lactic acids.

is expected. Although the activity of the immobilized enzymes sharply increased with higher pH values in both cases, a carrier solution of pH 9·7 was used throughout this work because of the instability of the binding of immobilized enzymes and of NAD^+ itself at higher pH values (Bergmeyer, 1974).

The effect of NAD^+ concentration is shown in Fig. 5. As the NAD^+ concentration in the carrier solution was increased, the peak height

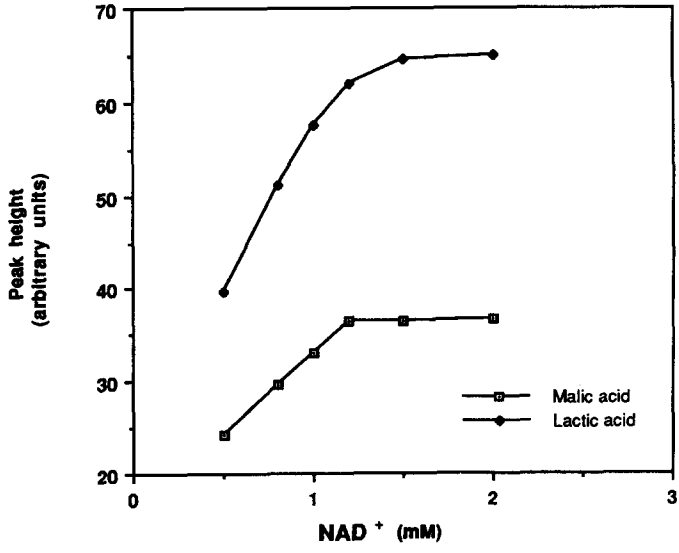


Fig. 5. Effect of NAD^+ concentration on the response to 0·5 mM malic and lactic acids.

increased linearly for both substrates at NAD^+ concentrations below 1·2 mM. The peak height was independent of NAD^+ concentration between 1·2 mM and 2 mM. Therefore, a carrier solution of 1·5 mM in NAD^+ was used throughout. The final constituents of the carrier stream were thus 0·05M carbonate–bicarbonate buffer, pH 9·7, also containing 1·5 mM NAD^+ and 1 mM EDTA.

The flow parameters were optimized by varying the carrier flow rate, the size of the injection loop and the lengths of the coils L_1 and L_2 (see Fig. 2).

Varying the flow rate will affect both the dialysis process and the enzymatic reactions. Figure 6 shows the effect, on the peak height, for both malic and lactic acid, of increasing the flow rate from 0·7 ml min^{-1} to 1·5 ml min^{-1}. As the flow rate increases, the analytical signal decreases for both substrates because the residence time in the dialysis unit is shorter. In another experiment without a dialysis unit the influence of the flow rate on the enzymatic reactions was studied. An increase in the flow rate did not modify the peak height obtained with 0·5 mM malic acid and 0·5 mM lactic acid. A final flow rate of 1 ml min^{-1} seems to be a good compromise between sensitivity and sample throughput.

With the optimized conditions, sample injection volumes ranging between 20 μl and 140 μl were investigated. The analytical signal rose with an increase in the injection volume for malic acid as well as for lactic acid. Therefore a 30 μl volume was chosen for future runs.

Finally, a study of the dimensions of the reaction coils for individual determinations of malic and lactic acid was performed. Variations of the L_1

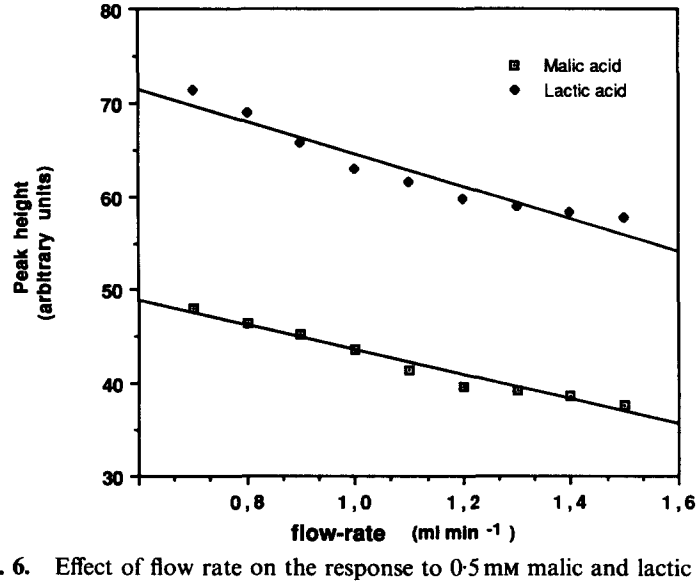


Fig. 6. Effect of flow rate on the response to 0·5 mM malic and lactic acids.

and L_2 lengths from 10 cm to 60 cm do not have any influence on the analytical signals obtained for either substrate.

System performance

With the optimal variables established (0·05 M carbonate–bicarbonate buffer pH 9·7, 1·5 mM NAD^+, 1 mM EDTA and 1 ml min^{-1} flow rate) for the individual determination of malic and lactic acid, the optimization of FIA variables—lengths of the channels (R_1, R_2 and R_3) and sample volume—was carried out for the simultaneous determination of these substrates (Fig. 1). A blank reactor positioned in channel R_1 was necessary for a good and reproducible division of the sample plug, avoiding the difference of pressure created by the enzymatic reactors inserted in channels R_2 and R_3.

The lengths of the channels were varied to obtain a good peak separation with maximal sample frequency. The optimized lengths for the three channels were 64 cm for R_1, 95 cm for R_2 and 185 cm for R_3. This study was performed with a rosé wine sample because the aqueous standards of malic acid and lactic acid do not have any peak corresponding to matrix interferences. Two individual standard solutions of malic and lactic acid, respectively, were used to determine the correspondence between peaks and substrate. When a real sample solution was injected into the FIA system, a train consisting of three peaks corresponding to each substrate appears in an FIA-gram. The first one corresponded to the fluorescence of the interferences of the wine itself, and its maximum value appeared at 44 s. The maximal signal for the second and third peaks appeared at 90 s and 225 s and corresponded to the fluoresence of NADH produced in the enzymatic reaction of malic acid and lactic acid, respectively.

Under these conditions, sample injection volumes between 20 μl and 140 μl were investigated. The peak height increased markedly with the injection volume. Therefore an 80 μl sample injection volume was selected (higher sensitivity and sampling frequency) and used for all future runs.

Features of the method

Under the optimized working conditions, malic and lactic acid were determined in the range 0·1–5 mM and 0·05–3 mM, respectively. The log–log calibration equations are given in Fig. 7. The reproducibility of this procedure was tested by twelve repetitive injections of a 1 mM malic and lactic acid standard solution. The relative standard deviations (r.s.d.) were 0·75% and 1·2% for malic and lactic acid, respectively, and were adequate for the requirements of the method. A sampling frequency of 15 h^{-1} for the simultaneous determination (30 determinations per hour) was obtained.

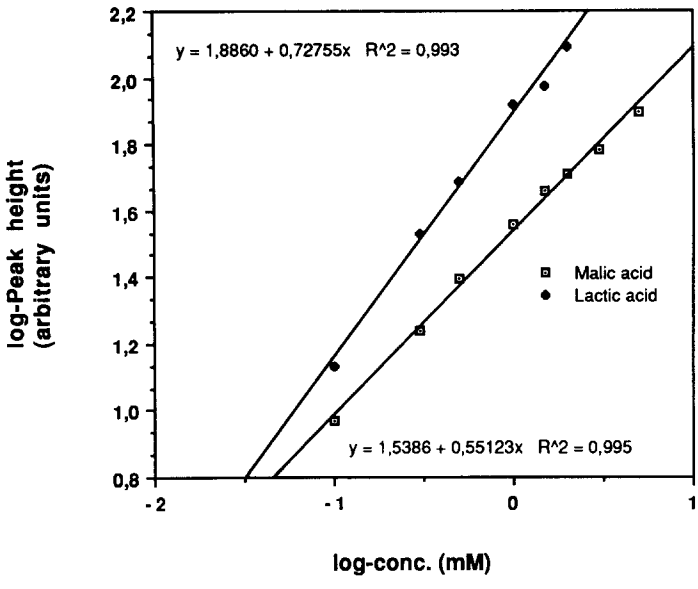


Fig. 7. Log–log calibration graph for malic and lactic acids.

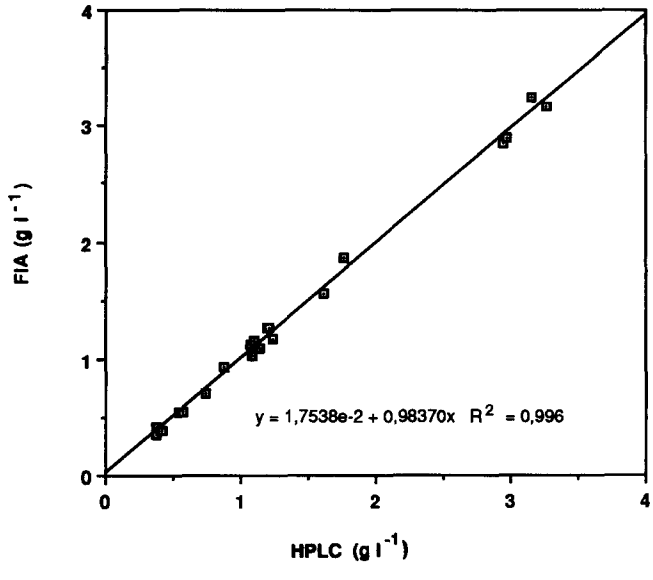
Interference study

Since wine is a very complex medium, a great number of interfering substances can occur. The effects of tartaric acid, fructose and SO_2 (100 mg litre^{-1}) were studied, no interference having been found when malic acid and lactic acid were determined by the proposed method. High concentrations of SO_2 (>150 mg litre^{-1}) give rise to a decrease in the signal, because they inhibited slightly the enzymatic reaction of these substrates. This offers no problem, as such high concentrations are rarely found in wines.

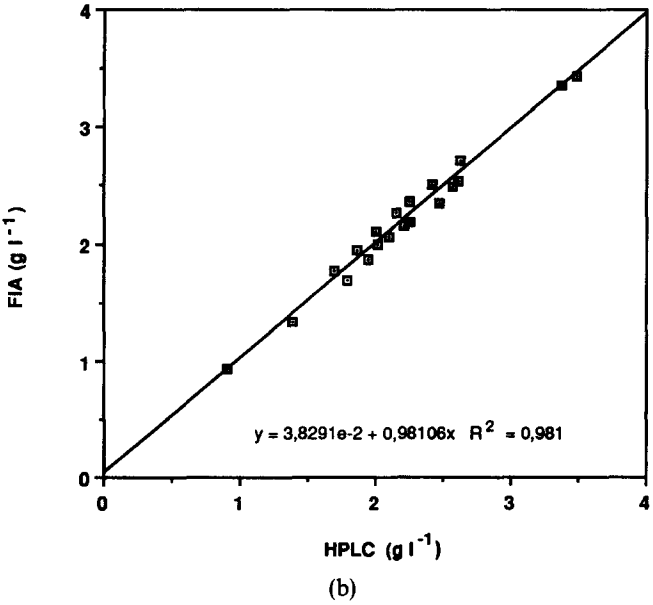
Application to wine samples—comparison with HPLC

In order to apply this method to the determination of malic and lactic acid concentrations, twenty different commercially available wines were analyzed. The wines (red, rosé and white) present a wide range of concentrations of these organic acids, because the malolactic fermentation is more or less developed. After the correction for the blank value, the amount of malic acid and lactic acid in these wines was calculated by means of the standard curves (Fig. 7).

The concentrations of L(−)malic acid and L(+)lactic acid in wine samples obtained by the immobilized enzyme-FIA method were compared with those obtained by the HPLC method (Fig. 8). The calculated linear



(a)



(b)

Fig. 8. Correlation of (a) malic acid and (b) lactic acid measurements in wines by FIA and HPLC methods expressed as g litre^{-1}.

regression and correlation coefficient for both malic acid (a) and lactic acid (b) indicated an excellent agreement between the results obtained by these two methods.

CONCLUSIONS

Interest in the measurement of malic and lactic acid is widely recognized. The enzymatic determination by a continuous flow method allows for maintaining the sensitivity and specificity of the enzymatic reaction and obtaining the results of the analysis quickly (30 determinations per hour). The accuracy of the described method is good and it is completely free from interferences.

The proposed flow-injection configuration solves the problem of the matrix contribution to the analytical signal by means of the introduction of a channel with a blank reactor.

This method is as effective as the reference HPLC method used but it is faster, consumes less reagent and no sample pretreatment is required, as the dialyzation itself produced the dilution of the sample.

The same L-MDH and L-LDH columns were used at 20°C during all work (six months) without any significant decrease in activity.

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