SIMULTANEOUS FLOW INJECTION ANALYSIS OF L-LACTATE AND L-MALATE IN WINE BASED ON THE USE OF ENZYMES

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The simultaneous determination of L-malate and L-lactate, by enzyme-supported FIA, was developed using two enzyme reactors in parallel and a single oxygen electrode. NADH formed in the reaction of malate dehydrogenase (MDH) was regenerated to NAD with dissolved oxygen, using vitamin K_3 and diaphorase (DI). L-Lactate was determined using the enzyme lactate oxidase (LOD). When sample solutions were simultaneously injected into the two reactors (the MDH-DI-reactor and the LOD-reactor) with a controlled residence time, a train of two peaks corresponding to L-lactate and L-malate were seen in the FIA-gram. The peak currents were linearly related to the L-malate and L-lactate concentration in the range 0.05-1.2 mM and 0.01-0.5 mM, respectively. The present system was applied to the determination of L-lactate and L-malate in white wine. The results showed a good agreement with those obtained using a conventional method (F-Kit method), suggesting that this system may be applicable to the monitoring of malo-lactic fermentation during wine production.

1. INTRODUCTION

Food is a complex mixture of many compounds, the concentrations of which are always changing. Therefore, high selectivity and rapidity is required for the analysis of food. Flow injection analysis (FIA) based on the use of enzymes is a suitable method available to meet this demand. This method has been used to determine specific compounds that effect the quality of food (1).

In some foods the concentration ratios of several ingredients can be an important index to the quality of that food. Therefore the analysis of such foods requires that the concentrations of these ingredients should be simultaneously determined.

This paper describes the simultaneous determination of L-malate and L-lactate in wine by using FIA, which includes a parallel configuration of two enzyme reactors and a single Clark oxygen electrode. L-Malate was determined using malate dehydrogenase (MDH) with diaphorase (DI) and L-lactate was determined using lactate oxidase (LOD) according to the methods reported previously (2,3).

2. MATERIALS AND METHODS

2.1 Reagents. CNBr-activated Sepharose 4B was obtained from Pharmacia LKB. Amino-Cellulofine was purchased from Seikagaku Kogyo Co. NAD was obtained from Kohjin Co. MDH (EC 1.1.1.37; from pig heart, 1200 U/mg) was obtained from Boehringer Mannheim GmbH, and DI (EC 1.6.99; from *Bacillus megaterium*, 30 U/mg) and LOD (EC number not available; from *Pediococcus* sp., 33 U/mg) were donated by Toyo Jozo Co. Vitamin K_3 (2-methyl-1,4-naphthoquinone) was purchased from Wako Pure Chemical Industry Co. L-Lactic acid (lithium salt) was from Sigma Chemical Co. All other chemicals were of analytical reagent grade and were used without further purification.

2.2 Enzyme immobilization. MDH (240 U) and DI (60 U) were immobilized on CNBr-activated Sepharose 4B (0.25 g dry weight) as reported previously (2), and was packed into a glass tube (2 mm i.d. x 10 cm). LOD (42 U) was immobilized on Amino-Cellulofine (0.3 g wet weight) as reported previously (4) and was packed into a glass tube (2 mm i.d. x 10 cm). These glass tubes were defined as the MDH-DI-reactor and the LOD-reactor, respectively.

2.3 Flow system. A schematic diagram of the flow system is shown in Fig.1. A teflon tube (1.0 mm i.d. and 2.0 mm o.d.) was used for the flow system throughout. The sample solution, diluted with 0.05 M pyrophosphate buffer (pH 9.0) and saturated with vitamin K_3, was pumped through the sample loop (150 μl) of the lactate line. This was then mixed with 5 mM NAD solution (prepared with the above buffer) before reaching another sample loop (150 μl), the malate line. The sample solutions were simultaneously injected into the two lines with a sixteen-way switching valve (Hitachi, K-1600) and transported to the enzyme

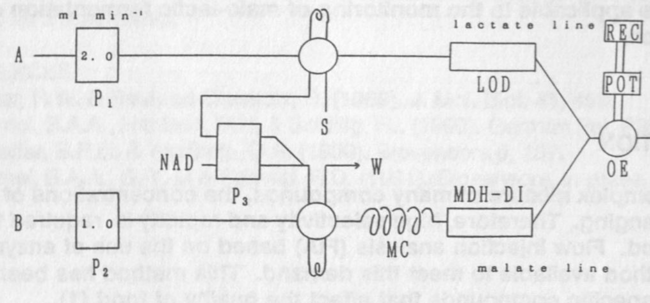

Fig. 1 FIA system for simultaneous determination of L-malate and L-lactate. A, 0.05 M pyrophosphate buffer (pH 9.0); B, 0.05 M pyrophosphate buffer (pH 9.0) saturated with vitamin K_3 ; P_1, P_2 and P_3, micro-tube pump; MC, mixing coil; OE, oxygen electrode; POT, potentiostat; S, sample solution; R, recorder.

reactors. After leaving the enzyme reactors the samples entered a flow-through cell equipped with a Clark oxygen electrode (Yellow Springs Instrument, Model No. 5331). When the residence time of the sample solutions (between the injection point and flow-through cell) were controlled, a train of two peaks corresponding to L-lactate and L-malate was seen in the FIA-gram. In the malate line, the mixing coil (80 cm) was set between the injection point and the MDH-DI-reactor. The potential of the oxygen electrode was fixed at -0.8 V. Amperometric measurements were made with a laboratory-made potentiostat and the peak heights were registered on a recorder (Rikadenki Ind. Co., DB101). The determinations were all carried out at 20 °C.

2.4 F-Kit method. Two F-Kits for L-malate and L-lactate were purchased from Boehringer Mannheim GmbH and the analyses were performed according to the manufacture's manual.

3. RESULTS AND DISCUSSION

When dehydrogenases are used in FIA, the addition of NAD is an important factor effecting the suitability of the system. In this paper, we have selected the automatic pre-mixing method shown in Fig.1. In this method NAD and sample solution are pumped at the same flow rate in the sampling lines, and transported to the sample loop of the malate line after mixing.

Experiments were first conducted to establish the optimum conditions for the detection of L-malate. When the flow rate was changed in the range 0.52 to 1.0 ml/min, no significant difference in the responses was observed. But a decrease in the time for baseline reversion was observed with increased flow rate. As the higher flow rate may cause a compaction of soft Sepharose gel, leading to a shorter life time for the reactor and a lowering of the reproducibility, we have selected 1.0 ml/min as the optimum flow rate. In order to allow sufficient mixing of the NAD solution with the sample solution, the mixing coil was set in the malate line. According to our previous paper (2), we have used 0.1 M pyrophosphate buffer (pH 9.0) as the carrier solution and 5 mM as the optimum NAD concentration.

The pH dependence of the LOD-reactor was studied from pH 6.0 to 9.0. No significant difference in the response was found in this range, suggesting that the reactor had a very broad pH optimum. From this result 0.05 M pyrophosphate buffer (pH 9.0) was used as the carrier solution, in agreement with that for the MDH-DI-reactor.

Using the FIA system for simultaneous determination of L-malate and L-lactate, it seemed reasonable to select the response for L-malate as the retarding peak because of the use of the mixing coil in the malate line. Figure 2 shows the effect of the flow rate in the lactate line on the separation of the two peaks during which the flow rate of the malate line was fixed at 1.0 ml/min. The flow rate ratio of the two lines affected the peak height and width of both peaks in addition to their separation. A good separation of the two peaks was achieved at a flow rate of 2.0 ml/min. Since the higher flow rate led to a lower peak height for L-malate, we have selected 2.0 ml/min as the optimum flow rate of the L-lactate line.

At the optimum conditions described above, the peak currents were linearly

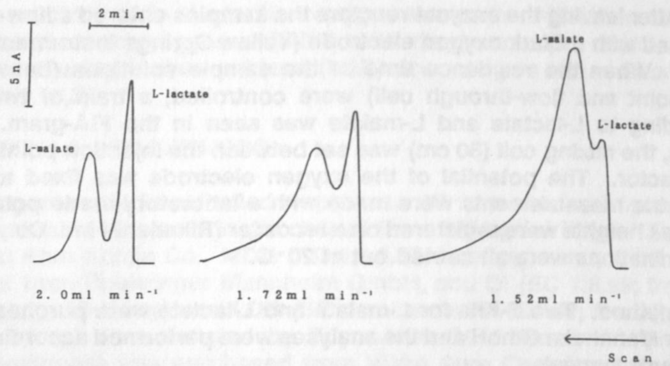

Fig. 2 Effect of flow rate in lactate line on response curves.
The flow rate in the malate line was fixed at 1 ml/min. *L*-malate concentration, 1.2 mM; *L*-lactate concentration, 0.5 mM.

related to the L-malate and L-lactate concentration in the range 0.05-1.2 mM and 0.01-0.5 mM, respectively. The relative standard deviations for five repeated injections was 0.92% and 1.0% for L-malate (0.6 mM) and L-lactate (0.2 mM), respectively. The sampling frequency was 15 samples/h.

Applying this method to the analysis of wines commercially available, a 40-fold dilution of each sample was required (5). Table 1 shows the results of the determination of L-malate and L-lactate in white wines. The results show a good agreement with those obtained using a conventional method (F-Kit method), suggesting that this system may be applicable to the monitoring of malo-lactic fermentation during white wine production.

Table 1 Comparison of the present method (FIA) with F-Kit method

Sample	L-malate/ mM		L-lactate/ mM	
	FIA	F-Kit	FIA	F-Kit
wine 1	15.9	15.2	6.69	6.59
wine 2	8.7	9.0	5.76	6.30
wine 3	23.1	23.1	3.72	3.66
wine 4	13.4	13.9	0.79	0.73
wine 5	6.5	6.6	0.61	0.58

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