Simultaneous Determination of Ethanol and Acetaldehyde in Liquor Using a Flow System Composed of Two Enzyme Reactors and an Octadecylsilica Column

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Simultaneous determination of ethanol and acetaldehyde was performed with an apparatus consisting of two enzyme reactors placed either side of an octadecylsilica column in a single flow line. The enzymes used were alcohol dehydrogenase for ethanol analysis, and aldehyde dehydrogenase for acetaldehyde analysis. The most favorable concentration of NAD^+ in the carrier for the simultaneous determination of ethanol and acetaldehyde was studied in wine or *sake*, in which the ethanol concentration was much higher that that of acetaldehyde. An NAD^+ concentration of 0.1 mM was adopted. To distinguish between NADH formed due to ethanol and that formed due to acetaldehyde, several buffers (pH 7.8) were also examined for use as the carrier medium, and triethanolamine buffer was found to be the most favorable. The calibration curve for acetaldehyde was linear (r^2 = 1.000) in the range of 0.2–100 μM. With respect to ethanol, plotting the logarithm of the peak area versus that of the concentration gave a linear relationship (r^2 = 0.997) in the range of 0.04–100 mM. This method was applied to the simultaneous analysis of ethanol and acetaldehyde in several types of liquor. The results were similar to those obtained using a commercially available test-kit method, suggesting the reliability and practicality of this method in analyzing real samples.

Key words —— enzyme reactor, flow injection analysis, simultaneous determination, liquor, octadecylsilica column

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INTRODUCTION

Flow systems using immobilized enzymes have been applied in the analysis of numerous enzyme substrates. Trends in recent studies include the introduction of an device for pretreatment to the flow system with an enzyme reactor: an electrodialysis unit,[1] a dialysis unit and anion exchange column[2] to exclude interfering substances, an Amberite XAD-4 column[3] to concentrate phenol in the atmosphere, a pervaporation unit for determination of volatile compounds,[4] and a separation column to separate analyts.[5]

Single substrates are generally analyzed using flow systems with immobilized enzymes. However, simultaneous determination of two substrates has also been carried out in designated systems by distinguishing the elution times of the two enzymatic products. In many cases, this distinction is performed using a delaying coil.[2,6–9] On the other hand, we have used an octadecylsilica (ODS) column[10,11] for separation of NADH formed in two different enzymatic reactions, and have performed simultaneous determinations of two reaction products.

In order to improve the determination of enological parameters, immobilized enzymes in a flow line were utilized for the simultaneous determination of ethanol and acetaldehyde in wines by Valcarcel *et al.*,[7] who overcame the differences in ethanol and acetaldehyde concentrations (the molar concentration of ethanol is three orders of magnitude larger than that of acetaldehyde). They used a long reaction coil and a sample plug with large volume in order to reduce the concentration of ethanol. However, the flow system was considerably complex, containing four flow lines. In the present report, we investigated the simultaneous determination of ethanol and acetaldehyde in liquor using a simple one-line flow system with two enzyme reactors placed either side of an ODS column, as was utilized in previous studies.[9,10] The difficulty with this system is that the signals for acetaldehyde and ethanol must be detected in a comparable scale despite the large difference in initial concentration.

Aldehyde dehydrogenase (ALDH) was used for analysis of acetaldehyde, while alcohol dehydrogenase (ADH) was used for analysis of ethanol. An ODS column was placed between the two enzyme reactors in order to separate the two NADH peaks

$$CH_3CHO + NAD^+ + H_2O \overset{ALDH}{\rightleftharpoons} CH_3COOH + NADH + H^+ \quad (1)$$

$$C_2H_5OH + NAD^+ \overset{ADH}{\rightleftharpoons} CH_3CHO + NADH + H^+ \quad (2)$$

formed in the respective reactions.

MATERIALS AND METHODS

Materials —— ALDH from yeast was purchased from Boehringer Mannhein (Germany); ADH was obtained from Oriental Yeast (Japan); and NAD^+, 25% aqueous solution of glutaraldehyde and aminopropyl glass (500 Å pore size, 200–400 mesh), was purchased from Sigma Chemical Co. (U.S.A.). The ODS column used was a Hitachi #3056 (4 mm i.d. × 50 mm).

Enzyme Immobilization —— ALDH was immobilized as described below. To aminopropyl glass beads (0.4 g) in 3.6 ml of 0.1 M phosphate buffer (pH 10.0), 0.4 ml of a 25% aqueous solution of glutaraldehyde was added. The mixture was bubbled with N_2 gas for 1 hr at room temperature. After the activated aminopropyl glass was filtered and washed with 100 ml of water, 3.2 ml of 0.05 M phosphate buffer (pH 6.0) and 40 units of ALDH were added. The mixture was stirred with a shaker for 20 hr at 4°C, and then bovine serum albumin was added to a final concentration of 1% (w/v), and the resulting mixture was shaken for 4 hr. Enzyme-immobilized glass beads were filtered and washed with 100 ml of 0.05 M phosphate buffer (pH 6.0) and then with 100 ml of water. Beads were stored in 3.2 ml of 0.05 M phosphate buffer (pH 6.0). The activity of the glass-immobilized enzyme was directly measured using a modified soluble enzyme assay method. The activity observed in the immobilized enzyme was 2.7 units (recovery 6.7%). ALDH-immobilized glass beads were packed into a stainless steel column (2 mm i.d. × 50 mm). Immobilization of ADH was also carried out as described above. Eighty-five units of ADH were immobilized from 2000 units of ADH (recovery 4.3%). The immobilized ADH was also packed into a stainless steel column (2 mm i.d. × 40 mm).

Apparatus —— Figure 1 shows a diagram of the apparatus used in the present study. Enzyme reactor 1 (ER1) denotes the enzyme reactor containing ALDH, and enzyme reactor 2 (ER2) is the reactor containing ADH. The ODS column was positioned between the two enzyme reactors. The enzyme reactors and the ODS column were immersed in a water bath (WB) thermostated at 30°C. The carrier (C) used was 0.1 M triethanolamine buffer (pH 7.8) containing 10 mM mercaptoethanol, 0.1 M KCl and 0.1 mM NAD^+. Carrier was delivered at a flow rate of 0.4 ml/min with a Shimadzu LC-10ADVP pump (P). NADH formed in the enzymatic reactions was detected at 340 nm by a Hitachi L-4200H spectrophotometer (D) with a 17.7 μl flow cell. The peak area was obtained using a Hitachi D-2500 data processor (DP) and is expressed in the unit of volt • sec. The sample injection volume was 50 μl.

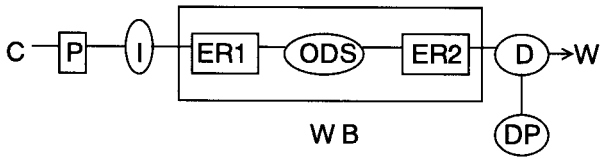

Fig. 1. Apparatus Used for Simultaneous Determination of Ethanol and Acetaldehyde

C, carrier reservoir; P, pump; I, sample injector; ER1, enzyme reactor containing ALDH-immobilized glass beads; ER2, enzyme reactor containing ADH-immobilized glass beads; ODS, octadecylsilica column; D, spectrophotometer; DP, data processor; WB, water bath; W, waste.

RESULTS AND DISCUSSION

The equilibrium of the ADH-catalyzed reaction [reaction (2)] lies to the left. This is a favorable characteristic for the simultaneous determination of acetaldehyde and ethanol in wine because the ethanol content is much larger than that of acetaldehyde. However, in order to perform the simultaneous analysis by a single injection of a diluted sample, ideal reaction conditions must be identified, under which the signal due to ethanol is substantially reduced in comparison with that due to acetaldehyde. Therefore, the dependence of the enzymatic reactions (1) and (2) on NAD^+ concentration in the carrier was studied.

The effects of the NAD^+ concentration on reaction (1) were examined using apparatus in which only ER1 was contained. The dependence of peak area of NADH formed by reaction (1) on NAD^+ concentration in a range from 0.02 to 0.5 mM is shown in Fig. 2. Peak area was almost constant at concentrations greater than 0.1 mM. Figure 2 also depicts the effects of NAD^+ concentration on reaction (2). The peak area increased almost linearly with increasing NAD^+ concentration, in contrast with that ob-

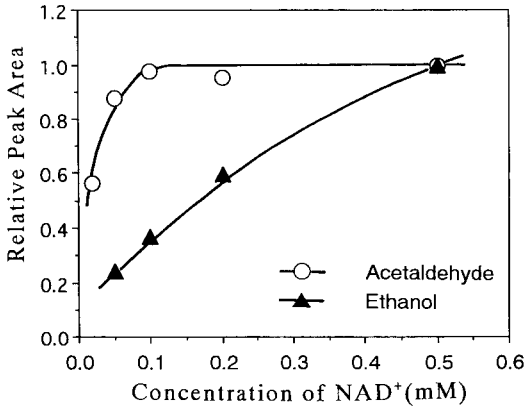

Fig. 2. NAD^+ Concentration Dependence of Enzymatic Reactions

Ordinate indicates peak area relative to that with 0.5 mM NAD^+. Substrate concentration in the sample was 50 μM acetaldehyde and 40 mM ethanol.

Table 1. Appearing Times of the Peaks due to Ethanol and Acetaldehyde with Various Carriers (Concentration; 0.1 M)

Buffer	Ethanol (min)	Acetaldehyde (min)
Pyrophosphate	4.3	17.0
Phosphate	4.2	14.4
Tris	4.1	13.9
PIPES	4.6	13.0
Triethanolamine	4.4	8.9
HEPES	4.5	7.1

The carrier used was 0.1 M buffer (pH 7.8) containing 10 mM mercaptoethanol, 0.1 M KCl and 0.1 mM NAD^+. Flow rate of the carrier was set at 0.4 ml/min. The sample used was a solution containing 20 mM ethanol and 10 μM acetaldehyde.

served for reaction (1). Based on these results, an NAD^+ concentration of 0.1 mM was used in the carrier in order to maximize peak area in acetaldehyde analysis and to produce appreciably decreased peak area in ethanol analysis. The mercaptoethanol and KCl in the carrier are essential for activation of ALDH, while mercaptoethanol reduces ADH activity to 80% of that observed in carrier containing no mercaptoethanol.

Various buffers were investigated for use as carriers to separate the two peaks for NADH formed in ER1 and ER2. Table 1 lists the appearing times of two peaks. The appearing time of the peak of NADH due to acetaldehyde was comparatively longer with a carrier composed of pyrophosphate, phosphate, Tris and PIPES buffer. The separation of the two peaks was not satisfactory in the case of HEPES. As

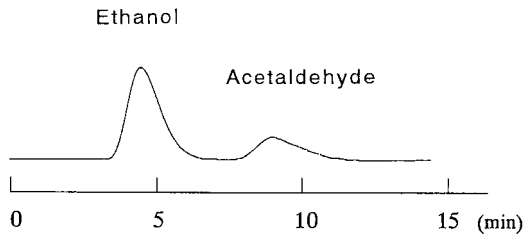

Fig. 3. Peaks for Ethanol and Acetaldehyde Observed When 0.1 M Triethanolamine Buffer (pH 7.8) was Used as a Carrier Buffer

Sample contained 10 μM acetaldehyde and 20 mM ethanol.

a result, triethanolamine buffer was selected for used as the carrier. Analysis of samples containing only acetaldehyde or only ethanol afforded only one peak, indicating that the ALDH and ADH reactors respectively utilized acetaldehyde and ethanol as substrates. Figure 3 shows the two peaks observed when triethanolamine was used as a carrier buffer. The peaks due to ethanol and acetaldehyde appeared at 4.4 min and 8.9 min, respectively. Samples containing both ethanol and acetaldehyde could be simultaneously analyzed within 13 min. The calibration curve for acetaldehyde was linear in the range of 0.2–100 μM (r^2 = 1.000) and the detection limit was 0.1 μM (S/N = 3). The calibration curve for ethanol did not show linearity when peak area was plotted against concentration. However, when the curve was plotted using logarithms of the peak area and concentration, linearity was seen in the range of 0.04–100 mM (r^2 = 0.997), with a detection limit of 0.02 mM. The relative standard deviation in the peak areas in a sample containing 20 μM acetaldehyde and 20 mM ethanol was 1.5% (n = 6) for acetaldehyde and 2.0% (n = 6) for ethanol. The activity of the ALDH reactor was almost constant after 21 days (140 measurements), but that of the ADH reactor was reduced to 63% of original activity after 26 days (120 measurements), over which period the enzyme reactors were stored at 4°C when not in use.

The present method was applied to analysis of acetaldehyde and ethanol in wine and sake, and the results are shown in Table 2. The ethanol content was about 2 M, while the acetaldehyde content was in the range of 0.3 to 1 mM, which was consistent with values previously obtained,[12] and considerably smaller than those reported by Valcarcel *et al.*[6](about 4 mM). The results obtained using the present method were in good agreement with those obtained using a commercially available test kit (F-kit, Boehringer Mannheim), thus indicating the reliabil-

Table 2. Ethanol and Acetaldehyde Content in Various Types of Liquor

	Ethanol (M)		Acetaldehyde (mM)	
	Present Method	F-kit	Present Method	F-kit
White wine 1	1.96	2.08	1.06	1.06
White wine 2	1.91	2.00	0.34	0.35
Red wine	1.89	1.91	0.53	—[a)]
Sake 1	2.39	2.42	0.47	0.48
Sake 2	2.36	2.50	0.52	0.53

Values shown are mean values of double determinations. Samples for analysis were prepared by diluting the original liquor 50-fold (for white wine 2, *sake* 1 and *sake* 2), or 100-fold (for white wine 1 and red wine) with carrier. *a*) Accurate measurement was not possible due to a color change which occurred when red wine was mixed with the F-kit (Boehringer Mannheim) buffer.

ity and practicality of this method for analyzing real samples.

The apparatus used in the present study is composed of a single line and is relatively simple. Simultaneous analysis of ethanol and acetaldehyde could be performed by a single injection of the sample, in contrast to the method of Varcarcel *et al.*[6)]

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