Flow Injection Analysis of Glycerol Based on Oxygen Consumption Using Glycerol Dehydrogenase and NADH Oxidase

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A flow injection analysis system for the determination of glycerol has been developed which incorporates a Clark-type oxygen electrode and an enzyme reactor packed with Sepharose 4B on which glycerol dehydrogenase and NADH oxidase are co-immobilized. The response of the system depended on the concentration of flavine adenine dinucleotide (FAD) added into the carrier solution [0.1 M glycylglycine-KOH buffer (pH 9.2) containing 30 mM ammonium sulfate]. A linear relationship between the glycerol concentration and the response current in the presence of 60 µM FAD was observed over the 0.1 - 1.5 mM range. The sampling frequency was about 25 samples/h at a flow rate of 0.5 ml/min, and the relative standard deviation was 0.65% at 2.5 mM glycerol level (n=10). Application of this system to commercial white and red wines gave satisfactory results compared with the F-kit method. No sample pretreatment was necessary other than dilution.

Keywords Oxygen electrode, NADH oxidase, flow injection analysis, glycerol determination

Glycerol is the most important by-product of yeast alcoholic fermentation, and thus a main constituent of wine. Therefore, the content of glycerol is closely related to the quality of wine.

Many methods, including high-performance liquid chromatography and gas chromatography, have been used for the determination of glycerol in wine.2,3 These methods, however, are generally time-consuming and require expensive instrumentation; sometimes a pretreatment procedure of the samples is also necessary.

In order to overcome these drawbacks of the conventional methods, flow injection analysis (FIA) methods for glycerol have been developed by utilizing a combination with immobilized enzymes.2-13 Most enzymatic FIA methods have been based on the use of glycerol dehydrogenase (GDH) or glycerokinase and glycerol-3-phosphate oxidase. Wines, especially red wines, contain relatively large amounts of polyphenol compounds, such as tannins.14 Since these compounds are reducing agents, they seriously interfere with some detection systems by amperometry, based on the use of a solid electrode. This may be the reason why only few of the enzymatic FIA methods previously developed have been utilized for wine analysis.2,4

We have applied NADH oxidase (NOD) from Bacillus licheniformis to an indication reaction for the dehydrogenases.15 The use of NOD has permitted the construction of an analytical system for dehydrogenase's substrate by a detection system based on a Clark-type oxygen electrode. Since the working electrode is protected against passivation in the solution by a gas-permeable membrane, an oxygen electrode is suitable for analyzing foods which contain a relatively high content of electroactive species. We developed the FIA system for ethanol in which immobilized alcohol dehydrogenase was combined with NOD, and have demonstrated its applicability to commercial white and red wines without having any pretreatment, except for dilution. The present paper describes a determination system of glycerol based on the use of a Clark-type oxygen electrode and the combined use of NOD and GDH (Fig. 1). This system was applied to the determination of glycerol in commercial red and white wines without using any pretreatment procedure. The results were compared with those obtained by the commercial enzymatic food-analysis kit (F-kit) method.
Experimental

Reagents

CNBr-activated Sepharose 4B was obtained from Pharmacia LKB. NAD was purchased from Kohjin Co. GDH (EC 1.1.1.6; from Enterobacter aerogenes, 500 U/ml) was obtained from Boehringer Mannheim GmbH and NOD (EC number not available; 60 units/mg protein) from Bacillus licheniformis was a gift from Marukin Shoyu Co.; it is also presently available from Nacalai Tesque Co. FAD was purchased from Nacalai Tesque Co. All other chemicals were of analytical grade and were used without further purification. Distilled and deionized water was used for all procedures.

Enzyme immobilization

GDH (10 IU) and NOD (15 or 30 units) were immobilized on CNBr-activated Sepharose 4B (0.20 g dry weight), as previously reported, and was packed into an acrylic tube (3 mm i.d. X 6.4 cm). The enzyme reactor packed with gel in which 10 IU of GDH and 15 or 30 units of NOD were immobilized are called Reactor 1 and Reactor 2, respectively. The enzyme reactor was stored in a 0.1 M phosphate buffer (pH 8.0) at 5°C.

Flow system

A schematic diagram of the flow system is shown in Fig. 2. A Teflon tube (0.86 mm i.d. and 1.4 mm o.d.) was used throughout the flow system. The standard solution of glycerol and the commercial wine sample (1:100) were diluted with a 0.1 M potassium phosphate buffer (pH 8.0). These solutions were mixed with an equivalent volume of a 5 mM NAD solution prepared using the same buffer; the mixed solution (20 µl) was injected into a carrier stream with a sample injector (Rheodyne, Model 9125). The carrier was pumped with a peristaltic pump (Gilson, Miniplus 3). The injected sample was pumped through the enzyme reactor to a flow-through cell (Denki Kagaku Keiki Co., FLC-41) equipped with a Clark-type oxygen electrode (Denki Kagaku Keiki Co.). The potential applied at the oxygen electrode was −0.7 V, amperometric measurements being made with a potentiostat (Denki Kagaku Keiki Co., IOL-50); the peak height was recorded as the response of a recorder (Graphitec, SR 6211). In the present paper, the concentration of glycerol in the standard and sample solution is indicated as that in the solution before being mixed with the NAD solution. Glycylglycine-KOH buffer (0.1 M, pH 9.2) containing 60 µM FAD served as a carrier solution (unless otherwise mentioned). The determinations were all carried out at 29±1°C.

F-kit method

The F-kit method for glycerol was purchased from Boehringer Mannheim GmbH and the analyses were performed according to the manufacturer's manual.

Results and Discussion

One of the typical assay solutions of GDH from Enterobacter aerogenes is a glycylglycine buffer containing ammonium sulfate. Since GDH is activated by NH₄⁺, the ammonium salt is usually added into the assay solution. In the optimization of the FIA system, the pH of the glycylglycine buffer was adjusted with KOH because K⁺ also activates the enzyme. When we examined the effect of the pH of the carrier solution on the response current for 2.5 mM glycerol in the 8.0–9.2 pH range, the maximum response current was observed at pH 9.2. For higher pHs than 9.2, carbonate buffer was used instead of a glycylglycine buffer because of the pKₐ (8.25). A response current having almost the same value was observed at pH 9.2 immediately after the carrier solution was changed from glycylglycine to the carbonate buffer. However, the response current gradually decreased along with the pumping time of the carbonate buffer through the enzyme reactor and, consequently, a constant response was not obtained. When the carrier solution was re-changed to the glycylglycine buffer, the response current was restored. Therefore, the decrease in the response current was reversible. Since Na⁺ used for the preparation of the carbonate buffer inhibits the GDH reaction, the presence of the ion may have caused the reversible decrease in the response current. This tendency was also recognized for a pH higher than 9.2. From these results, a glycylglycine-KOH buffer (pH 9.2) was used as the optimum carrier solution in a subsequent experiment.

Figure 3 shows the effect of the ammonium sulfate concentration added into the carrier solution on the response current. When using an enzyme reactor in which only GDH was immobilized, Matsumoto et al. observed that the addition at the 30 mM level resulted in about a 10-fold increase in the response current compared with that in a carrier solution containing no ammonium salt. However, no dependence was recognized in the present FIA system. The difference may have resulted from the co-immobilized NOD that can recycle NADH formed in the GDH reaction in the present system. Since the ammonium ion improves the stability of the immobilized GDH, the salt was added at a concentration of 30 mM in further experiments.

Figure 4 depicts the effect of the concentration of NAD
on the response current. For a higher concentration of NAD than 4 mM, there was no dependence of the concentration of the response current. We have therefore chosen 5 mM as the optimum concentration.

When the flow rate was changed in the range 0.25 to 1.0 ml/min, both the response current and the time for a baseline reversion decreased along with an increase in the flow rate. The choice of the flow rate involves a compromise between the sensitivity and the sampling rate. A flow rate of 0.5 ml/min was used in a subsequent experiment, considering the relatively high response and short sample output time.

FAD acts an activator for NOD used in the present work. Figure 5 shows the effect of the FAD concentration in the carrier solution on the response current using two kinds of enzyme reactors (Reactor 1 and Reactor 2). As shown in the figure, a rapid increase in the response current was observed up to 30 µM and a tendency to level off was recognized above this concentration in both enzyme columns. Reactor 2, which had a greater amount of NOD, showed a smaller ratio of the response current at 30 µM to that at 0 µM FAD, compared with the ratio of Reactor 1. This suggests that the dependence of FAD on the response current can be controlled by adjusting the amount of immobilized GDH and NOD, and that the use of a higher amount of NOD against GDH may permit one to construct an analytical system without requiring an exogenous addition of FAD. Taking into account this possibility, we used not only a carrier solution containing a high concentration of FAD (60 µM), but also one not containing FAD in further experiments.

Under the conditions described above, and in both of the reactors, a linear relationship between the glycerol concentration and the response current was recognized in the 0.1 - 1.5 mM and 0.25 - 1.0 mM ranges in both the
presence and absence of FAD, respectively (Fig. 6). The relative standard deviation for ten successive determinations at the 2.5 mM level glycerol was 0.65%, and the sampling frequency was about 25 samples/h.

The stability of the immobilized enzymes was estimated for 65 d by the use of a carrier solution containing FAD. No tendency for the response current of 2.5 mM glycerol to decrease was recognized by 25 d (the total operational time and injection time by that day were 12 h and 150 injections, respectively); the response current was then gradually decreased. The relative response current at the 65th day was 60%, compared with that at the first day after immobilization (total operational time, 18 h; injection numbers, 235 injections). Therefore, the stability of the enzyme reactor in the present work was comparable to that in other previously reported systems.3

Table 1 shows the results of a determination of glycerol in four kinds of commercial wines. The results obtained by the present method showed 6 to 14% higher values than those obtained by the F-kit method. A similar result was also seen in a previous paper by Matsumoto et al., in which only GDH was used in the enzymatic FIA system.4 The F-kit method is an enzymatic spectrophotometric method using glycerokinase, pyruvate kinase, lactate dehydrogenase in the free state with ATP and NADH used as co-factors. The reason why the present method gave slightly bigger values may have been due to a difference in the substrate specificity between GDH in the present FIA system and glycerokinase in the F-kit method. As pointed out by Puchades et al., GDH oxidizes not only glycerol (1,2,3-propanetriol), but also 1,2-propanediol and 1,2-butanediol, which are present in several products, including wine.2 Furthermore, ethanol present at a high concentration in wine is also oxidized at a 1% relative rate of glycerol.17 It would therefore be likely that these compounds present in wine cause a slightly positive error in the results. Although a more precise comparison is required with regard to the concentration of 1,2-propanediol, 1,2-butanediol and ethanol in each wine sample, a determination using the present system compared relatively well with the F-kit method overall. The fact that no difference in the results was recognized between white and red wines suggests that the polyphenols present in red wine failed to interfere with the determination by the present FIA system. In this experiment, a carrier solution containing no FAD was used as well as that containing 60 µM FAD (Table 1). No difference in the value between them was recognized, meaning that the enzyme reactor (Reactor 2) is applicable to the determination of glycerol in wine samples, even in the absence of FAD.

### Table 1 Comparison of the present FIA method with a conventional method (F-kit method)

<table>
<thead>
<tr>
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<th>FIA, w/v%</th>
<th>F-kit, w/v%</th>
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<tbody>
<tr>
<td>0 µM FAD</td>
<td>0.40</td>
<td>0.39</td>
</tr>
<tr>
<td>60 µM FAD</td>
<td>0.82</td>
<td>0.80</td>
</tr>
<tr>
<td>Red wine 1</td>
<td>0.55</td>
<td>0.53</td>
</tr>
<tr>
<td>Red wine 2</td>
<td>0.59</td>
<td>0.60</td>
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In both methods, these samples were diluted to 100-fold with 0.1 M phosphate buffer (pH 8.0).

### References


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