Determination of Formate Using Immobilized Formate Dehydrogenase in a Flow System and Its Application to Analyze the Formate Content of Foodstuffs

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The quantity of formate was determined using apparatus comprised of a reactor with immobilized formate dehydrogenase (FDH) in the flow line. NADH formed by an enzymatic reaction was fluorometrically detected. The optimal concentration of NAD^+ in the carrier was determined. The maximum peak area due to NADH was observed at pH 7.0 when the pH of the carrier consisting of piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) buffer ranged from 6.0 to 8.0. Various buffer types were also examined as carrier media at pH 7.0 and PIPES buffer showed the maximum peak area. When the carrier composed of PIPES buffer (0.1 M, pH 7.0) was used, the calibration curve for formate was linear in the range of 0.5–50 μM (r = 1.000). Relative standard deviations of the peak area at 1 μM and 10 μM were 2.6% (n = 7) and 1.6% (n = 7), respectively. This method was applied to the analysis of formate in foodstuffs, and formate content determined by this method agreed with that determined by a commercially available test-kit.

Key words —— formate, formate dehydrogenase, immobilized enzyme, flow injection analysis, foodstuff

INTRODUCTION

Various methods for the determination of formic acid have been proposed, including gas chromatography, high-performance liquid chromatography (HPLC) and enzymatic methods. However, these methods are time-consuming and require troublesome operations. In the analyses by gas chromatography, formic acid must be transformed to its derivatives, methyl formate,[1–4] dimethyl formamide,[5] and formanilide.[6] The steps for the separation or extraction are also needed, or the headspace method is frequently utilized. In the HPLC method,[7,8] the derivatization of formic acid with a long reaction time must be carried out prior to analysis. In the enzymatic methods utilizing formate dehydrogenase (FDH), formic acid itself is analyzed. Thus, its derivatization is not needed, differing from the methods mentioned above. However, the enzymatic reaction using soluble enzymes[9–13] requires troublesome operations and a comparatively long reaction time. We have utilized immobilized enzymes in a flow system[14–17] for the determination of the component of drinks. In these methods, enzymes can be repeatedly used. Due to enzyme specificity, only one peak corresponding to the substrate is usually observed at a short time after injection of a sample. Thus, many samples can be analyzed in a constant time.

In this work, formate was determined using a reactor containing immobilized FDH in the flow system. Formic acid is used as a food preservative,[11] thus, the present method was applied to determine formic acid in foodstuffs, vinegar, wine and honey.

The enzymatic reaction[11] is shown below. In this reaction, FDH (E.C.1.2.1.2) is utilized. NADH formed by the reaction was detected fluorometrically.

$$HCOOH + NAD^+ \xrightarrow{FDH} CO_2 + NADH + H^+$$

MATERIALS AND METHODS

Materials —— FDH and a 25% aqueous solution of glutaraldehyde were purchased from Sigma Chemical Co., Ltd. (St. Louis, MO, U.S.A.), NAD^+ was from the Oriental Yeast Co., Ltd. (Tokyo, Japan), formic acid and 2-amino-2-hydroxymethyl-1,3-propanediol(Tris) were from Wako Pure Chemicals Industries Ltd. (Osaka, Japan), piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) and 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) were from Dojindo Laboratories (Kumamoto, Japan) and aminopropyl glass (500 Å pore size, 200–400 mesh) was from CPG, Inc. (Lincoln Park, NJ, U.S.A.). F-kit is a test kit from Roche Diagnostics GmbH (Basel, Switzerland).

Enzyme Immobilization —— FDH was immobilized[14,15] as described below. To aminopropyl

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glass beads (0.4 g) in 3.6 ml of 0.1 M phosphate buffer (pH 10.0) was added 0.4 ml of a 25% aqueous solution of glutaraldehyde. 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 water, 20 units of FDH was added with 3.2 ml of 0.05 M phosphate buffer (pH 6.0). The mixture was stirred with a shaker for 20 hr at 4°C, and shaken for a further 4 hr after bovine serum albumin (BSA) was added to 1% (w/v). 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. They were stored in 3.2 ml of 0.05 M phosphate buffer (pH 6.0). Seven percent of the activity of the initially applied enzyme was found in the immobilized enzyme. The enzyme-immobilized glass beads thus prepared were packed into a stainless steel column (4 cm, i.d. 2 mm).

Apparatus —— The apparatus used in this study is the same as that shown in Fig. 1 in the previous paper.[17] The enzyme reactor (ER) containing immobilized enzyme was immersed in a water bath (WB) at 30°C. The carrier used was 0.1 M PIPES buffer (pH 7.0) containing 1.0 mM NAD^+, and this was delivered at a rate of 0.4 ml min^{-1} by a Shimadzu LC-10AD pump (P). NADH formed by the enzymatic reaction was fluorometrically detected (λ_{ex}, 340 nm; λ_{em}, 460 nm) by a Hitachi F-1050 spectrofluorometer (D) with a 12 μl flow cell. The peak area was obtained by a Hitachi D-2500 data processor (DP). The sample injection volume was 50 μl.

Preparation of the Samples —— Vinegar (10 ml) was neutralized to pH 7–8 with potassium hydroxide solution, quantitatively transferred to a 20 ml volumetric flask, filled with water to the marked line and mixed. This solution was 50 times diluted with carrier. The solution, 100 times diluted from the original vinegar, was used for analysis. Wine was 2 times diluted with water, and the solution was 50 times diluted with carrier. Wine-1 was degassed prior to dilution with water. One gram of honey was weighed and transferred to a 10 ml volumetric flask with water. Then the flask was filled with water to the marked line and mixed. This solution was 50 times diluted with carrier for analysis.

Determination of Formate by F-kit —— All of the absorbances of the solutions described below were measured at 340 nm. Five minutes after mixing NAD^+ and the sample containing formate in potassium phosphate buffer (pH 7.5), the absorbance of the mixture was measured (E_1). FDH was then added to the mixture. Absorbance (E_2) was also measured 20 min after FDH addition. The values of E_2 and E_1 were respectively calibrated with a blank test. The content of formate was calculated from the difference of E_2 minus E_1 calibrated.

RESULTS AND DISCUSSION

The dependence of the NAD^+ concentration in the carrier upon the peak area due to NADH was ex-

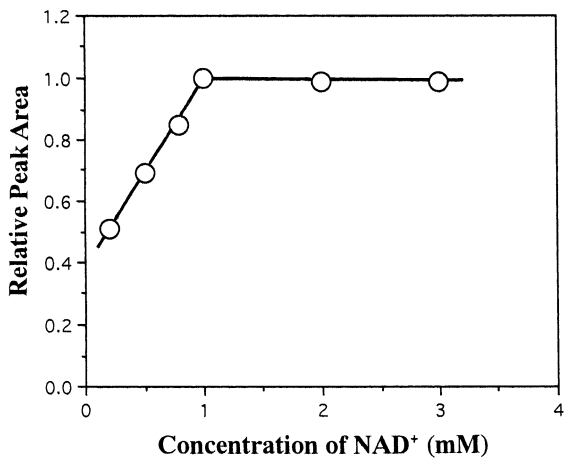

Fig. 1. Dependence of the Peak Area upon the NAD^+ Concentration of the Carrier

Ordinate indicates the peak area relative to that at 1.0 mM NAD^+. Values were obtained from the averages of triplicate determinations. Carrier was 0.1 M PIPES buffer (pH 7.0) containing NAD^+. Flow rate of the carrier was 0.4 ml min^{-1}. Temperature of the water bath in which ER was immersed was 30°C. Concentration of formate injected was 10 μM.

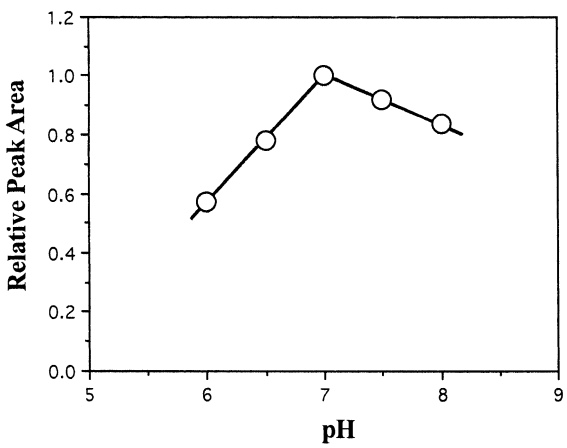

Fig. 2. Dependence of the Peak Area upon the Carrier pH

Ordinate indicates the peak area relative to that at pH 7.0. Values were obtained from the averages of triplicate determinations. Carrier used contained 1.0 mM NAD^+. Other conditions were the same as those described in the legend of Fig. 1.

Table 1. Effects of Various Buffer Types (0.1 M, pH 7.0) as Carrier Media upon the Peak Area due to NADH

Buffer	Relative peak area
HEPES	0.70
Phosphate	0.87
PIPES	1.00
Triethanolamine	0.18
Tris	0.50

Values are the peak areas relative to that obtained with PIPES buffer, and are the averages of triplicate determinations. Carriers contained 1.0 mM NAD^+. Other conditions were as described in the legend of Fig. 1.

Table 2. Formate Contents of Foodstuffs (μM)

Sample	Present method	F-kit method
Vinegar-1	409 ± 13	407
Vinegar-2	926 ± 15	920
Wine-1	77 ± 7	72
Wine-2	99 ± 3	84
Honey	53 ± 3 (μg)	54 (μg)

Values are the averages of triplicate determinations. The content of formate in honey is represented as μg per gram of honey.

amined. As shown in Fig. 1, NAD^+ concentrations of more than 1.0 mM afforded an almost constant peak area. Consequently, a concentration of 1.0 mM was used in the subsequent experiments.

Figure 2 shows the dependence of the peak area upon the pH of the carrier comprised of PIPES buffer in the pH range from 6.0 to 8.0. The maximum peak area was obtained at pH 7.0. Various buffer types were also examined as carrier media. Table 1 shows the effect of each buffer type (0.1 M, pH 7.0) as a carrier medium upon the peak area of NADH. Of the buffers, PIPES afforded the maximum peak area, whereas triethanolamine buffer showed a considerably smaller peak area. PIPES buffer (0.1 M, pH 7.0) was used as the carrier medium for the following experiments.

The peak due to formate was observed at about 1.9 min under the experimental conditions. The calibration curve obtained was linear (r = 1.000) in the formate concentration range from 0.5 to 50 μM, but concentrations less than 0.2 μM gave comparable peak areas. Thus the lower limit of detection was 0.5 μM, which is larger than that in the previous study,[17] 0.03 μM. The detection limits in gas chromatography were 10 μM[2] (flame ionization detector) and 5 μM[4] (mass spectrometry), respectively. That in HPLC method detecting benzimidazole, the reaction product of *o*-phenylenediamine with formic acid, at 267 nm was 0.1 μM[8] (S/N = 2), though the lower limit of linearity was 1.6 μM. And those in the enzymatic methods using soluble enzymes were 5 μM (fluorometric detection)[10] and 10 μM (spectrophotometric detection),[11] respectively. Thus, the lower detection limit in the present method is comparable with, or lower than those in other methods. As described above, the concentrations less than 0.2 μM afforded comparable areas in the present method. The detection limit would be improved, assuming that the calibration curve in this concentration range shows a linearity by using other buffer type than PIPES as a carrier medium.

The relative standard deviations of the peak area in the present method were 2.6% (n = 7) and 1.6% (n = 7) at 1 μM and 10 μM, respectively and these values are considered to be appropriate for the analysis of formic acid.

In the present method, only one peak appears at 1.9 min and the analysis of a sample finishes in 5 min. This is in contrast to other methods as gas chromatography or HPLC, where the analysis of the second sample can start only after all peaks of the first sample appeared completely. Thus, many samples can be analyzed in a constant time by the present method.

The present method was applied to analyze the formate content of vinegar, wine and honey. Table 2 lists the results of this analysis compared with those obtained by a commercially available test kit (F-kit), which showed good agreement.

After analysis of the samples, the carrier in the immobilized enzyme column was replaced by 0.05 M phosphate buffer (pH 6.0) and the column was stored at 4°C to prevent the decrease of enzyme activity; however, the peak area for 10 μM formate decreased to 85% of that initially detected after the analysis of 140 samples, including standard samples for a calibration curve. Thus, the immobilized enzyme used in the present study seemed to be less stable than the previously used immobilized enzymes.[16,17]

REFERENCES

1) Czerwiński, W. and Stepień, A. (1978) Gas chromatographic determination of small amounts of formic acid in mixtures containing phenol, acetone and aromatic hydrocarbons. *J. Chromatogr.*, **148**, 219–222.

2) Abolin, C., MacRae, J. D., Tozer, T. N. and Takki, S. (1980) Gas chromatographic head-space assay of formic acid as methyl formate in bioloigic fluids: potential application to methanol poisoning. *Biochem. Med.*, **23**, 209–218.
3) Lee, X. -P., Kumazawa, T., Kondo, K., Sato, K. and Suzuki, O. (1999) Analysis of methanol or formic acid in body fluids by headspace solid-phase microextraction and capillary gas chromatography. *J. Chromatogr. B*, **734**, 155–162.
4) Kim, J. K., Shiraishi, T., Fukusaki, E. and Kobayashi, A. (2003) Quantitation of formate by solid-phase microextraction and gas chromatography-mass spectrometry utilizing a [^{13}C]formate internal standard. *J. Chromatogr. A*, **986**, 313–317.
5) Bricknell, K. S. and Finegold, S. M. (1978) Improved method for assay of formic acid by gas-liquid chromatography. *J. Chromatogr.*, **151**, 374–378.
6) Umeh, E. O. (1970) Determination of formic acid in dilute aqueous solution and separation and determintion of aniline and toluidine isomers by gas chromatography of formanilide and formyl toluidides *J. Chromatogr.*, **51**, 139–146.
7) Vainiotalo, S., Pfäffli, P. and Zitting, A. (1983) Sensitive liquid chromatographic method for the determination of formic acid in air samples. *J. Chromatogr.*, **258**, 207–211.
8) Ohmori, S., Sumii, I., Toyonaga, Y., Nakata, K. and Kawase, M. (1988) High-performance liquid chromatographic determination of formate as benzimidazole in biological samples. *J. Chromatogr.*, **426**, 15–24.
9) Triebig, G. and Schaller, K.-H. (1980) A simple and reliable enzymatic assay for the determination of formic acid in urine. *Clin. Chim. Acta*, **108**, 355–360.
10) Makar, A. B. and Tephly, T. R. (1982) Improved estimation of formate in body fluids and tissues. *Clin. Chem.*, **28**, 385.
11) Schaller, K.-H. and Triebig, G. (1985) Formate. In *Methods of Enzymatic Analysis*, Volume VI, 3rd Edition, (Bergmeyer, H. U., Bergmeyer, J. and Grassl, M., Eds.), VCH Publishers, Weinheim, pp. 668–672.
12) Grady, S. and Osterloh, J. (1986) Improved enzymic assay for serum formate with colorimetric endpoint. *J. Anal. Toxicol.*, **10**, 1–5.
13) Buttery, J. E. and Chamberlain, B. R. (1988) A simple enzymatic method for the measurement of abnormal levels of formate in plasma. *J. Anal. Toxicol.*, **12**, 292–294.
14) Mori, H., Kogure, M., Kuroda, Y., Nissato, M., Tamura, Y., Watanabe, N. and Yamamoto, H. (1999) Simultaneous determination of glucose and sucrose using a flow system with two enzyme reactors and an octadecylsilica column in one line. *Anal. Lett.*, **32**, 1531–1541.
15) Mori, H. (1999) Simultaneous determination of D-lactic acid and L-lactic acid using a flow system with two enzyme reactors and an octadecylsilica column in one line. *Anal. Lett.*, **32**, 1301–1312.
16) Mori, H., Okamoto, Y. and Fujita, Y. (2005) Determination of isocitrate using immobilized isocitrate dehydrogenase in a flow system and its application to analyze the total isocitrate content of beverages. *J. Health Sci.*, **51**, 711–714.
17) Mori, H., Yamashita, A. and Maitani, S. (2007) Determination of L-malate using immobilized malate dehydrogenase and aspartate aminotransferase in a flow system and its application to analyze the L-Malate content of beverages. *J. Health Sci.*, **53**, 128–131.