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# DETERMINATION OF NEUTRAL FAT IN MILK BY AMPEROMETRIC FLOW INJECTION ANALYSIS WITH IMMOBILIZED ENZYME REACTORS

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## ABSTRACT

A neutral fat sensor, in which lipase and esterase were co-immobilized on Aminopropyl-Controlled-Pore Glass and glycerol dehydrogenase on Amino-Cellulofine, was developed for the continuous determination of neutral fat in milk. This sensor involved two steps of enzyme reactions. At first, lipase and esterase hydrolyzed neutral fat to glycerol and fatty acids. Next, glycerol dehydrogenase catalyzed the oxidation of glycerol produced to dihydroxyacetone using NAD. NADH produced by this enzyme reaction was monitored amperometrically on a platinum electrode. Linear relations between the square of sensor responses and triolein concentration were observed in the ranges of $1.28 \times 10^{-2}$-$8.53 \times 10^{-2}$ % with correlation coefficient larger than 0.99. The relative standard deviation for ten measurements of $8.5 \times 10^{-2}$ % triolein was 0.87 %.

## INTRODUCTION

Enzymatic method was applied for determination of neutral fat in milk. Neutral fat indicates monoglyceride, diglyceride and triglyceride, and all of them are ester of glycerol and fatty acids. Most of fats in foods are triglycerides and commercial cooking oils and fats are almost composed of triglyceride. The fats in milk comprised glyceride of many kinds of fatty acids, mainly saturated fatty acids such as palmitic, stearic and myristic acid, and unsaturated fatty acid such as oleic acid.

The Roese-Gottlieb method and the Babcock method have been used to determine fat in milk, and various procedures are available to determine neutral fat. Recently enzymatic methods have been described (1-2). These methods, however, are complicated and require relatively long time, additional reagents and expensive enzymes. The combination of immobilized enzyme reactors and the flow injection analytical method constitutes a rapid, simple and economical technique for the determination of neutral fat.

In this paper, lipase (EC 3.1.1.3) and esterase (EC 3.1.1.1) were co-immobilized on Aminopropyl-Controlled-Pore Glass (CPG) and glycerol dehydrogenase (EC 1.1.1.6) on Amino-Cellulofine. Lipase, esterase and glycerol dehydrogenase catalyze the reactions shown in Eqs. (1) and (2). The produced NADH in eq. (2) is monitored amperometrically.

$$\text{Triglyceride} \xrightarrow{\text{Lipase + Esterase}} 3\ \text{Fatty acids} + \text{Glycerol} \quad (1)$$

$$\text{Glycerol} \xrightarrow{\text{Glycerol dehydrogenase}} \text{Dihydroxyacetone} + \text{NADH} + \text{H}^{+} \quad (2)$$

## MATERIALS AND METHODS

Reagents. Lipase (LP. EC 3.1.1.3. from Chromobacterium viscosum) was obtained from Toyo Jozo Co.,Ltd.. Esterase (ER. EC 3.1.1.1. from Porcine Liver) was purchased from Sigma Chemical Co.. Glycerol dehydrogenase (GDH. EC 1.1.1.6. from Enterobactor aerogenes) was obtained from Boehringer Mannheim GmbH. Triolein (used as the standard triglyceride), albumin, (bovine, CRG-7), sodium cyanoborohydride and glutaraldehyde (25%) were purchased from Nacalai Tesque Inc. (Kyoto. Japan). Aminopropyl-Controlled-Pore Glass (CPG 120/200 mesh) was purchased from Electro Nucleonics, Inc. and Amino-Cellulofine was from Seikagaku Kogyo Co.(Tokyo Japan). Triton X-100 was purchased from Ishizu Pharmaceutical Co., Ltd. (Osaka, Japan). Triglyceride G-Test Wako was purchased from Wako Pure Chemical Industries, Ltd. All other chemicals were of reagent grade and were used without further purification.

### Preparation of LP and ER immobilized on CPG

LP (3100U) and ER (253U) were co-immobilized on CPG (0.18g

dry weight) as reported previously (3). CPG was washed thoroughly with 0.2M carbonate buffer (pH 10) on a sintered glass filter (G-3). The CPG thus purified was used as a support. The support was mixed with 0.2M carbonate buffer (pH 10) containing 5% glutaraldehyde for 2hr at 20℃ while shaking. The resulting precipitate was separated from the solution by filtration (sintered glass filter, G-3) and washed thoroughly with distilled water and 0.1M phosphate buffer (pH 7). By these procedures, aldehyde functionalities were introduced at the terminal $NH_2$ groups of the support. The support thus treated was put into 1ml of 0.1M phosphate buffer (pH 7, coupling solution) containing lipase and esterase and then allowed to react for 30 min at 5℃ while being shaken intermittently. The support obteined was reduced with about 2mg of sodium cyanoborohydride for 12hr at 5℃ and then washedthoroughly with 0.1M phosphate buffer (pH 7) conteining 0.5M NaCl (washing solution) and coupling solution in turn. The residual functionalities on the LP and ER co-immobilized CPG were then blocked with 1ml of 15mg/ml glycine solution by standing for 2hr at 20℃, and the LP and ER co-immobilized CPG was separated by filtration and was successively washed with the washing solution and the coupling solution. The LP and ER co-immobilized CPG was packed in a glass tube (2 mm i.d.× 10 cm) and the reactor was filled with the coupling solution and was stored at 5℃. This glass tube was defined as the LP-ER-reactor.

Preparation of GDH immobilized on Amino-Cellulofine

GDH (33U) was immobilized on Amino-Cellulofine (0.35g wet weight) as reported previously (3) and was packed into a glass tube (2 mm i.d. × 10 cm). This glass tube was defined as the GDH-reactor.

Preparation of sample, triolein emulsion and standard solution

To 40ml of the 6% BSA aqueous solution was added 0.52g of triolein. Then, the mixture was homogenized by the homogenizer (PHYSCOTRON, drive unit; NS-50, generator shaft; NS-20M, Nichion Irikakiki Ltd.) with dial 80 for 10 minute (5 min.× 2, interval10 min.).

The triolein emulsion was diluted with 0.125M carbonate buffer (pH 9.5) containing 30mM ammonium sulfate. Triton X-100 (1%) was added to the mixture. This solution was used as a standard solution.

Sample was also preparated in a similar manner.

Flow system

A schematic diagram of the flow system is shown in Fig. 1. The working solution (carrier solution) in a reservoir was propelled by a micro-tube pump (Tokyo Rikakikai Co., Ltd.) through an air-damper, a sample injection valve (16-way switching valve, Hitachi K-1600), and reactors of the LP-ER-,

and the GDH-reactor, and then was transported to an electrochemical flow-through cell and finally to a waste tank. The main flow line was divided into two flow lines at position S in Fig. 1 by a 3-way switching valve. The switching valve was properly switched to an immobilized GDH- reactor for glycerol signal intake or to a blank column for a blank value intake. The blank column was the same size as the enzyme column and contained Amino-Cellulofine without the immobilized enzyme. The sample flow system consisted of another micro-tube pump connected to the same sample injection valve (16-way swicthing valve) equipped with sample loops. The sample and co-enzyme NAD were injected by a sandwich method, which was reported previously (3). The design of the flow-through cell was mentioned previously (4).

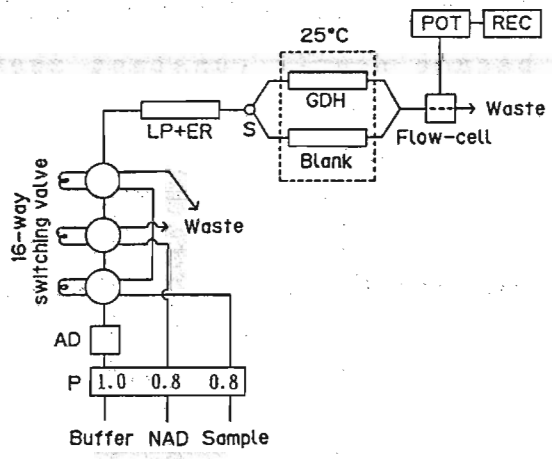


Figure 1. Schematic Diagram of the Flow Injection Analytical System.
P, microtube pump; AD, air-damper; LP+ER, immobilized LP and ER column; GDH, immobilized GDH column; Blank, blank column; S, 3-way switching valve; POT, potentiostat; REC, recorder; Buffer, 0.125M carbonate buffer containing 30mM ammonium sulfate (pH 9.5). The flow rates are indicated (in ml/min) in the pump (P).

Amperometric measurements were made with a laboratory-made multichannel potentiostat, which was described previously (5). The potential was set to be +0.75V vs. Ag/AgCl. Other instrument in the system were the same as those in our previous work (6).

## RESULTS AND DISCUSSION

### Amount of LP and ER

The effects of the amounts of LP and ER on the response of the reactor were investigated and shown in Figs. 2 and 3. In the case of LP, the response increased up to $1.7 \times 10^{4}$ U/g-dry gel and became nearly constant above this amount. In the case of ER, the response was maximum when this enzyme was not contained and became nearly constant above this amount.

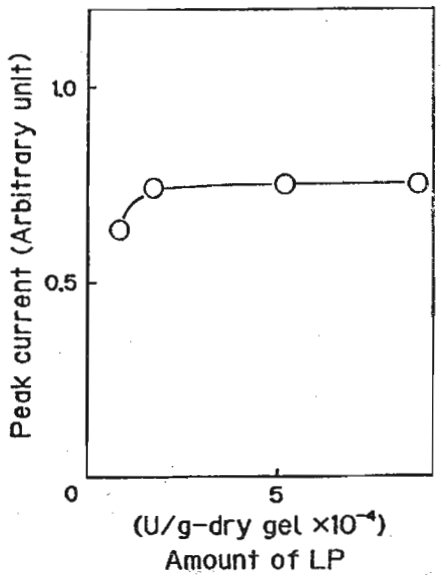


Figure 2. Effect of LP amount on peak current

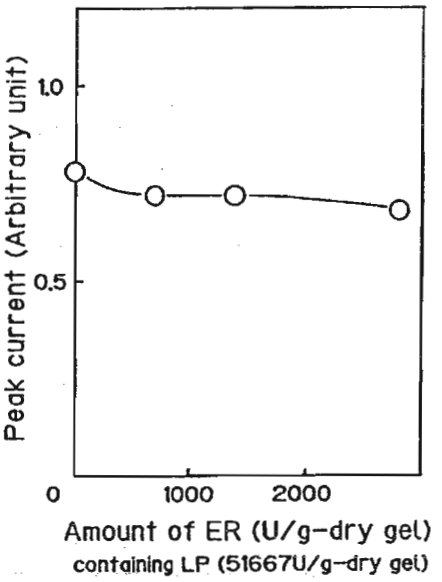


Figure 3. Effect of ER amount on peak current

Therefore, only LP $1.7 \times 10^4$ U/g-dry gel was immobilized on CPG in subsequent experiment.

pH and temperature dependence

The pH dependence of the LP-reactor was studied from pH 8.5 to 10.5, using 0.125M carbonate buffer containing 30 mM ammonium sulfate. The result is shown in Fig. 4. The optimum pH was between 9.5 and 10.0. The effect of the temperature on the response was also investigated in the range of 27- 60℃. As shown in Fig. 5, the response was nearly constant up to 45 ℃ and increased above this temperature and was maximum at 55 ℃. However, the response at 45 ℃ was about 88% compared to the maximum. It was reported that the native LP was stable below 40℃ (7,8). Therefore, subsequent work was done at 27 ℃, considering the long-term stability.

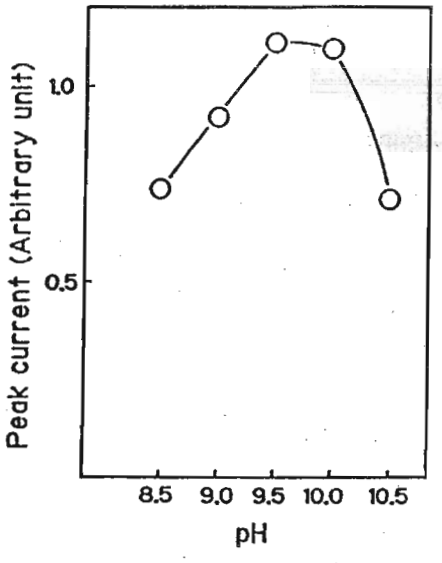


Figure 4. Effect of pH on peak current

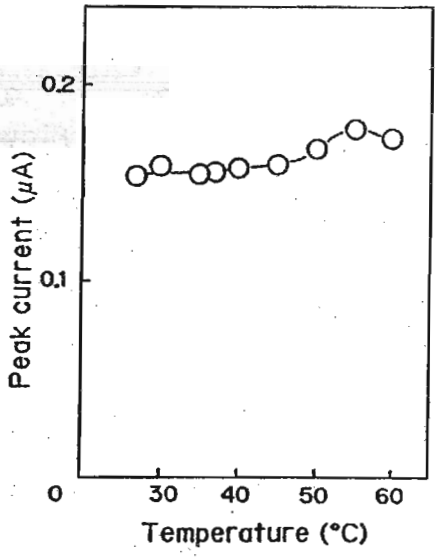


Figure 5. Effect of temperature on peak current

Flow rate

Figure 6 shows the effects of the flow rate on the sensor response. The response increased with decreased flow rate. The time for baseline reversion, however, was decreased with increased flow rate. A flow rate of 1.0 ml/min was used in this experiment, considering the response and the relatively short sample output time.

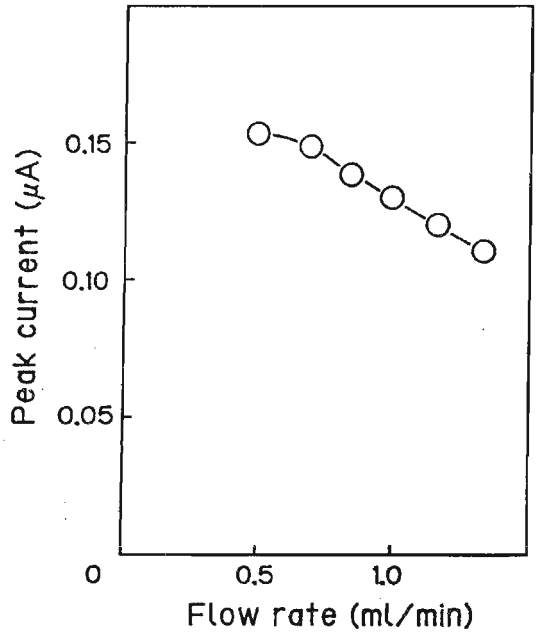


Figure 6. Effect of the flow rate on peak current

Effect of Triton X-100

The effects of the Triton X-100 concentration in the sample on the response of the reactor were investigated and shown in Fig. 7. It was reported that the Triton X-100 played important role for the activation of the reaction of lipase. The response

increased with increasing concentration, but the blank value also increased gradually. Therefore, the net response increased was maximum at 1% Triton X-100. Subsequent studies were done with 1% Triton X-100 in the sample.

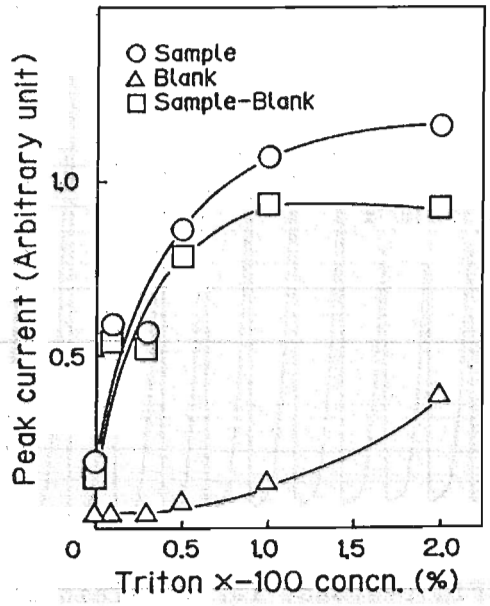


Figure 7. Effect of Triton X-100 concentration in sample on peak current.
○, sample; △, blank; □, sample-blank

Calibration, reproducibility and long-term stability

A typical calibration curve showed a slightly convex curve between response and triolein concentration ($1.42 \times 10^{-2} - 2.56 \times 10^{-1}$%) but linear relations between the square of sensor responses and triolein concentration were observed in the ranges of $1.28 \times 10^{-2} - 8.53 \times 10^{-2}$ % with correlation coefficients larger than 0.99. Figure 8 showed the typical response curve. The current increased rapidly just after injection of sample, and returned to baseline within 6 min. The relative

standard deviation for ten measurements at 0.085% level was 0.87%. One column of immobilized LP could be operated for at least one week, and, when not in use, stored at 5 ℃ in the working solution.

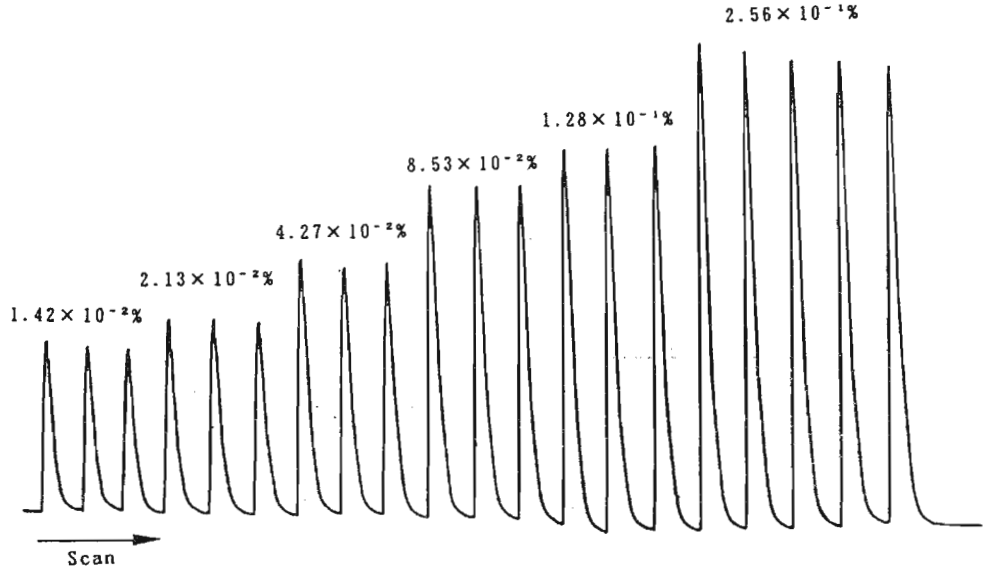


Figure 8. Typical FIA response for triolein standard solution.

Application to milk analysis

Table 1 shows the results for the determination of neutral fat in milk using of this system. Milk samples were diluted

Table 1. Comparison of the present method (FIA) with the G-test method for the determination of neutral fat in milk

| Sample | Present method (FIA),% (A) | G-test method % (B) | Bias % (A-B) |
|---|---|---|---|
| Milk 1 | 3.45 | 3.34 | 0.11 |
| Milk 2 | 3.51 | 3.59 | -0.08 |
| Milk 3 | 3.65 | 3.47 | 0.18 |
| Milk 4 | 3.34 | 3.57 | -0.23 |

90-fold and injected. The small responses from the blank column were subtracted from those from the GDH reactor. These results are compared to those obtained by the Triglyceride G-Test Wako (G-Test method) which is glycerol-3-phoshate oxidase/p-chlorophenol method to determine triglyceride. As shown in Table 1, the values measured with this FIA coincided relatively well with those of the G-Test method. This method is thought to be useful for simple and rapid measurement of neutral fat in milk.

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