Flow Injection Analysis of Short-Chain Fatty Acids in Milk Based on a Microbial Electrode

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A flow injection analysis system for the determination of short-chain fatty acids was developed incorporating a microbial electrode based on an oxygen electrode and *Arthrobacter nicotiana* immobilized behind a dialysis membrane. The system showed a high selectivity for short-chain fatty acids ($C_{4:0}$–$C_{12:0}$), and the system response was linearly related to the concentration of butyric acid over the range 0.11–1.7 mM. The sampling frequency was approximately 20 samples/h at a carrier flow rate of 1.0 mL/min. The microbial electrode was both highly selective and stable. The application of this system to determination of short-chain fatty acids in milk is described, and the results obtained are compared with those obtained by gas chromatography. The present system was applied to the monitoring of lipolysis of raw milk during storage.

INTRODUCTION

The rancid or off-flavor in milk and milk products is caused by the liberation of short-chain fatty acids ($C_{4:0}$–$C_{12:0}$) by milk or bacterial lipases (Kuzdzal-Sovoie, 1980; Walstra and Jenness, 1984). Therefore, the detection of short-chain fatty acids is very important in the quality evaluation of milk and milk products, in particular in connection with presently adopted methods of bulk milk collection and prolonged storage of raw milk.

The quantitative determination of free fatty acids has been carried out by titrimetric determination of total acidity or by colorimetric analysis based on the transfer of metal soaps from a copper or cobalt nitrate triethanolamine reagent, after the extraction of the free fatty acids into organic solvents (Dole and Meinertz, 1960; Itaya and Ui, 1965). These methods show no specificity related to fatty acid chain length. As short-chain fatty acids represent about 5–6% of the total fatty acid, their presence or absence has little influence on the final value. The determination of free fatty acids by gas chromatography (de Jong and Bading, 1990; Deeth et al., 1983) enables the determination of each fatty acid, but the quantitative recovery of short-chain fatty acids depends to a great extent on the method used for isolating the free fatty acids from the sample; the procedure is generally complicated. Shimizu et al. have proposed the enzymatic determination of free fatty acids (Shimizu and Yamada, 1985; Hosaka et al., 1981) based on monitoring the hydrogen peroxide formed by the two sequential reactions of the enzymes acyl-CoA synthetase and acyl-CoA oxidase. This method, which is selective and rapid, has been used to develop a sensor system for free fatty acids (Sode et al., 1989). In the application of this method to detect the rancidity of dairy products, specificity would be a problem. The acyl-CoA oxidase commercially available generally has a high specificity for long-chain and a low specificity for short-chain fatty acids, especially butyric acid (Shimizu and Yamada, 1985; Hosaka et al., 1981).

Wagner (1991) found that the microorganism *Arthrobacter nicotiana* exhibited acyl-CoA oxidase activity with a high specificity for short-chain fatty acids and suggested that this microorganism may be applicable to the determination of short-chain fatty acids. In this paper, we describe a rapid, convenient, and selective method for the determination of short-chain fatty acids using this microorganism combined with flow injection analysis and its applicability to the analysis of milk. The format of flow injection analysis was chosen because liquid handling and sample pretreatment by dilution can easily be automated, resulting in reproducible experimental conditions.

MATERIALS AND METHODS

Chemicals. Casein (sodium salt, from bovine milk) and dialysis membrane [cellulose tubing that retained 90–99% of a cytochrome *c* (MW 12 400) solution over a 10-h period] were obtained from Sigma Chemical Co. Poly(vinyl alcohol) (PVA; MW ca. 22 000) was purchased from Serva Feinchemica Co. Other reagents were of analytical grade. Deionized water was used in all procedures.

Culture and Immobilization of the Microorganism. *A. nicotiana* (DSM 6707, Braunschweig, Germany) was cultured under aerobic conditions at 30 °C for 18 h in a 100-mL flask containing 25 mL of a medium composed of 0.3% yeast extract, 0.5% peptone, 0.5% K_2HPO_4, 0.5% KH_2PO_4 (all w/v), and 1% butyric acid (v/v). The medium was centrifuged at 4800 rpm for 10 min, and the cell pellet was washed with approximately 100 mL of 0.9% sodium chloride solution and allowed to dry in the refrigerator for 1 h. Forty milligrams of the dried cell mass was mixed with 1 mL of 0.1 M potassium phosphate buffer (pH 7.0) containing 5% PVA. The cell suspension (1 mL) was spread on a glass plate (5 cm × 2 cm) and dried at room temperature for 3 h and then overnight at 5 °C. The resulting membrane had a thickness of approximately 50 μm.

Assembly of the Microbial Electrode. The microbial electrode consisted of the microorganism membrane, a dialysis membrane, and an oxygen electrode (Schott Geräte GmbH) (Figure 1). The PVA membrane with the immobilized microorganism was cut into a circle (5.9-mm diameter) and fixed over the Teflon membrane of the oxygen electrode. The dialysis membrane was used as a protective membrane to cover and fix the microorganism membrane to the electrode.

Free fatty acids were added to 0.1 M potassium phosphate buffer containing 3% (w/v) casein, and this solution was emulsified using ultrasonic treatment (10 min). This served as the sample solution. The emulsion was stable for 1 day.

FIA System. A schematic diagram of the flow system is shown in Figure 2. Potassium phosphate buffer (0.1 M, pH 7.0) served as the carrier solution. Sample solution (35.5 μL) was injected into the carrier solution, pumped through a mixing coil (0.8 mm i.d., 79 cm long), and transported to a flow-through cell equipped with the microbial electrode. The mixing coil was integrated in the FIA system to provide an internal dilution of the sample. The length was suitable for an efficient mixture of milk samples with the carrier. When the flow rate was set at 1.0 mL/min, the

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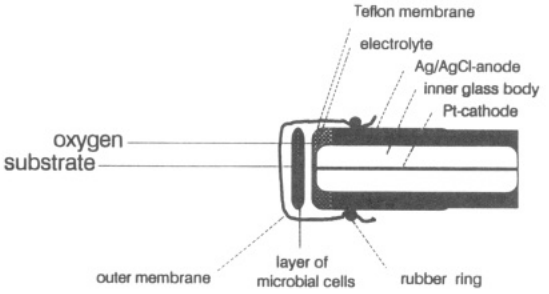

Figure 1. Schematic diagram of the microbial electrodes.

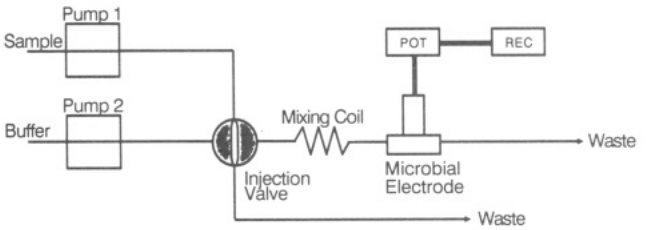

Figure 2. Schematic diagram of the flow injection analysis system. POT, potentistat; REC, recorder.

dispersion ratio of the injected sample solution was about 8. The measurements were carried out at 30 °C, and the peak height was recorded as the electrode response. For the analysis of milk using the FIA system, the milk sample was directly injected without dilution unless otherwise indicated. The concentration of short-chain fatty acids in milk was calculated using a calibration curve for butyric acid or caprylic acid.

Determination of Free Fatty Acids in Milk by Gas Chromatography. Free fatty acids were extracted from commercially available or agitated raw milk with ether/heptane (1:1 v/v) containing ethanol and sulfuric acid followed by isolation using a weak anion exchanger (Supelco, Supelclean LC-NH_2 SPE tube) according to the method of de Jong and Bading (1990). A HP 5890 gas chromatograph (Hewlett-Packard) equipped with a flame ionization detector was used with a fused silica capillary column, 30 m × 0.53 mm i.d., coated with 5% phenylmethyl silicon (df = 0.88 μm) (Hewlett-Packard). The carrier gas (nitrogen) flow rate was 6 mL/min. During analysis the oven temperature was raised from 40 to 260 °C at a rate of 6 °C/min.

RESULTS AND DISCUSSION

The proposed sensor comprises a microorganism membrane, a protective membrane, and an oxygen electrode. It was applied to the analysis of milk. Milk is a complex mixture of many compounds, in which the casein content is characteristically very high. Therefore, the first criterion for selecting a suitable protective membrane was that the membrane pore size be small enough to prevent the casein molecule from reaching the microbial membrane. A dialysis membrane with a cutoff limit of about 12 000 was therefore chosen as the protective membrane.

Optimization of the FIA System. The pH dependence of the microbial electrode was tested over the pH range 6.0–7.5 (Figure 3). Although the sensitivity gradually increased with a decrease in pH, the preferred buffer was pH 7.0 due to the higher stability of the microorganism at this pH. When the flow rate was varied over the range 0.5–2.0 mL/min, both the response and the time required for reversion to baseline (90%) decreased with increasing flow rate (Figure 4). The choice of flow rates involves a compromise between sensitivity and sampling frequency. When a flow rate of 1.0 mL/min was used, the response of the sensor was linear between 0.11 and 1.7 mM *n*-butyric acid (Figure 5) with a correlation coefficient of 0.9991 ($y = 23.11x + 1.4358$; in arbitrary units), and the precision was better than 5% on five successive determinations.

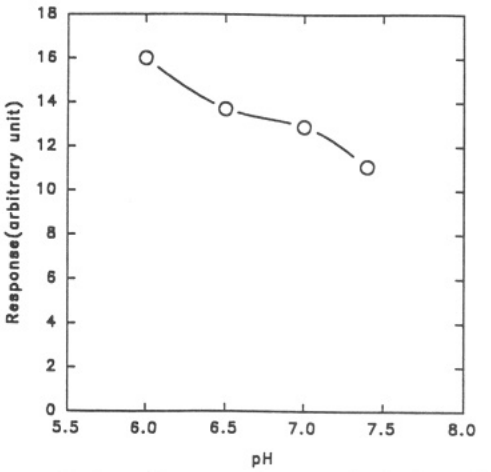

Figure 3. pH dependence of the microbial electrode. The substrate used was butyric acid with a concentration of 1.68 mM; the flow rate in the FIA was 1.0 mL/min.

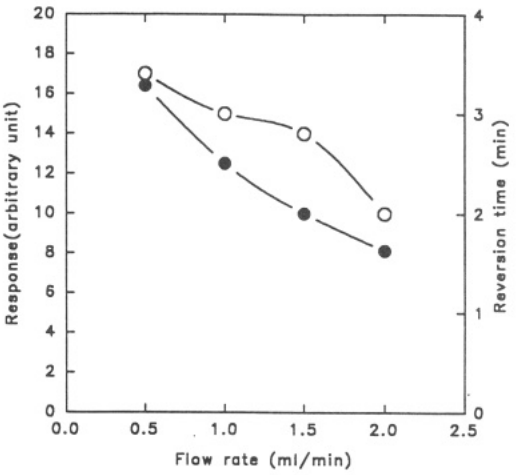

Figure 4. Effect of flow rate on response (●) and the time required for reversion to baseline (○).

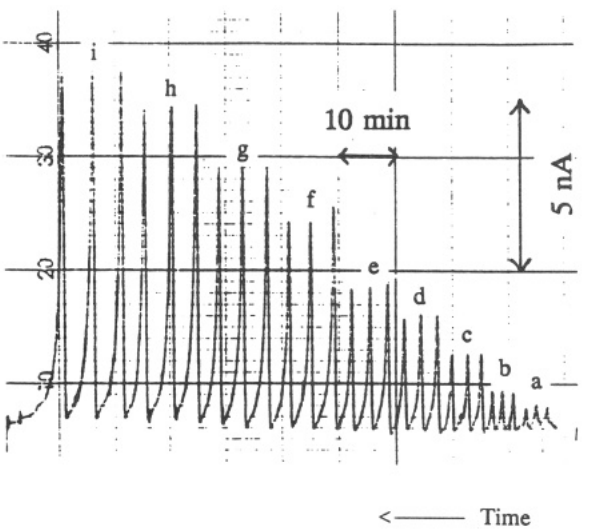

Figure 5. Typical response curve for butyric acid: (a) 0.11 mM; (b) 0.28 mM; (c) 0.56 mM; (d) 0.84 mM; (e) 1.12 mM; (f) 1.68 mM; (g) 2.24 mM; (h) 2.80 mM; (i) 3.36 mM.

The sampling frequency was approximately 20 samples/h.

Optimum Immobilization Conditions. Figure 6 shows the effect of cell concentration in the PVA membrane on the system response. The maximum response was observed with a cell concentration between 2 and 4

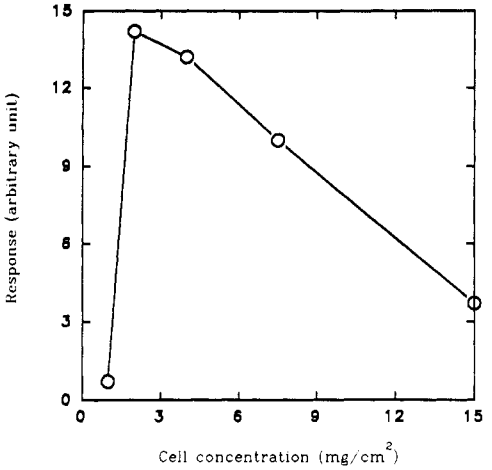

Figure 6. Effect of the amount of cells incorporated in the PVA membrane on the system response (experimental conditions as in Figure 3).

Table I. Comparison of Responses for Various Fatty Acids

fatty acid	notation	relative value, %	fatty acid	notation	relative value, %
acetic acid	2:0	26	myristic acid	14:0	9
butyric acid	4:0	59	palmitic acid	16:0	1
caproic acid	6:0	62	stearic acid	18:0	1
caprylic acid	8:0	100	oleic acid	18:1	1
capric acid	10:0	80	linoleic acid	18:2	6
lauric acid	12:0	47			

mg/cm². At a high concentration of microorganisms, the response decreased drastically. Therefore, a cell concentration of 4 mg/cm² was selected for further experiments.

Specificity of Response for Fatty Acids. Sugiura et al. (1977) and Sode et al. (1989) prepared standard solutions by mixing free fatty acids and buffer containing bovine serum albumin as a model of human blood. In this study standards were prepared by mixing fatty acids and buffer containing casein, the main protein found in milk. Table I shows a comparison of the response for various kinds of fatty acids present in milk. The sensor showed a high specificity for short-chain fatty acids from butyric acid ($C_{4:0}$) to lauric acid ($C_{12:0}$). These fatty acids are liberated in milk by bacterial lipases and are mainly responsible for the rancid flavor of dairy products (Walstra and Jenness, 1984). This result suggests that this sensor may be applicable to the detection of rancid flavor in dairy products.

Selectivity. Any specific compound with a high diffusion rate through the dialysis membrane and a high assimilation by the immobilized microorganism could show a response in this system. Therefore, the selectivity of the microbial electrode was examined by using the following compounds (the concentration used is given in parentheses): casein (3%), bovine serum albumin (0.04%), lactose (4.6%), galactose (10 mg %), glucose (10 mg %), L-glycine (3 mg %), choline (30 mg %), creatine (3 mg %), urea (30 mg %), L-lactate (3 mg %), citrate (0.2 %), β-lactoglobulin (0.5 %), and triolein (1%). The concentration examined was the usual level found in milk (Walstra and Jenness, 1984). No response was observed for these compounds. The reason might be that the present method is based on the use of a microbial electrode covered with a dialysis membrane with a small pore size and set in the continuous flow stream. Thus, the diffusion resistance may be too high to allow diffusion through the dialysis membrane and assimilation by the microorganisms within the short residence time of the injected sample.

Table II. Recovery of Added Fatty Acids into Milk

fatty acid	concn, mM added	concn, mM present	concn, mM found	recovery, %
butyric acid	0	0.65	0.65	
	0.28	0.93	0.88	95
	0.56	1.21	1.20	99
	1.12	1.77	1.69	95
	2.80	3.45	3.39	98
capric acid	0	0.41	0.41	
	0.29	0.70	0.71	101
	0.58	0.99	0.98	99
	1.45	1.86	1.86	100

Table III. Comparison of the Present Method with Gas Chromatographic Analysis of Short-Chain Fatty Acids in Milk

	fatty acid	milk 1,[a] μmol/L	milk 2,[a] μmol/L	milk 3,[a] μmol/L
GC analysis	$C_{4:0}$	69 (41)	190 (112)	194 (114)
	$C_{6:0}$	76 (47)	94 (58)	111 (69)
	$C_{8:0}$	16 (16)	71 (71)	78 (78)
	$C_{10:0}$	151 (121)	8.7 (7.0)	5.6 (4.5)
	$C_{12:0}$	95 (45)	75 (35)	198 (93)
	total	407 (270)	439 (283)	587 (359)
microbial sensor[b]		410	435	589

[a] The values in parentheses are equivalents of the $C_{8:0}$ fatty acid based on the relative activity of the microbial sensor toward the different fatty acids (Table I). [b] The concentrations are given in equivalents of the $C_{8:0}$ fatty acid.

Application of the Microbial Electrode. The microbial electrode was applied to the determination of short-chain fatty acids in milk. Known amounts of butyric acid or capric acid were added to a commercially available milk sample. As shown in Table II, satisfactory recovery data (95–101%) were obtained. Table III shows a comparison of the present method with gas chromatography for the determination of short-chain fatty acids in commercial and agitated raw milk. The concentration of short-chain fatty acids in milk by the microbial sensor was calculated using the calibration curve for caprylic acid ($C_{8:0}$), which was the fatty acid that showed the highest sensitivity. The concentration of each short-chain fatty acid obtained by gas chromatography was normalized to the concentration of caprylic acid using the sensitivity ratios obtained from Table I. The results obtained by the microbial sensor were remarkably higher than the sums of the normalized concentration of $C_{4:0}$–$C_{12:0}$ obtained by gas chromatography. This may be due to the specificity of the microbial sensor to other fatty acids or to effects resulting from the mixture of microbial substrates.

Figure 7 shows the relationship between the storage time of agitated raw milk at 25 °C and the concentration of short-chain fatty acids calculated from the sensor response. The sensor response increased with the storage time of the raw milk, which suggests that the present system may be applicable to the monitoring of the lipolysis of raw milk during storage.

Storage Stability of the Immobilized Microorganism and Operational Stability of the Microbial Electrode. Three microbial electrodes were prepared under the same conditions (Figure 8). The response of the first electrode was determined on the day of preparation. The remaining electrodes were stored in a nutritional medium containing butyric acid at 5 °C prior to use. With electrodes used on the third and seventh day following their preparation, no decrease in response was observed, compared with the response obtained on the first day. Thus, the electrodes could be stored for at least 7 days without loss of activity (solid circles in Figure 8).

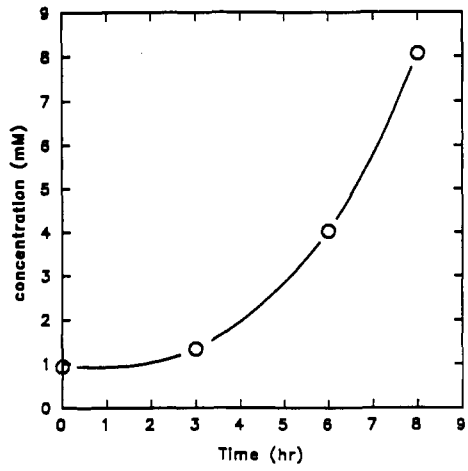

Figure 7. Relationship between storage time of agitated milk at 25 °C and sensor response. The concentration of short-chain fatty acids was calculated by using a calibration curve for butyric acid. The raw milk was diluted 5- and 10-fold with 0.1 M potassium phosphate buffer (pH 7.0) for applying to the FIA system after 6 and 8 h of storage, respectively.

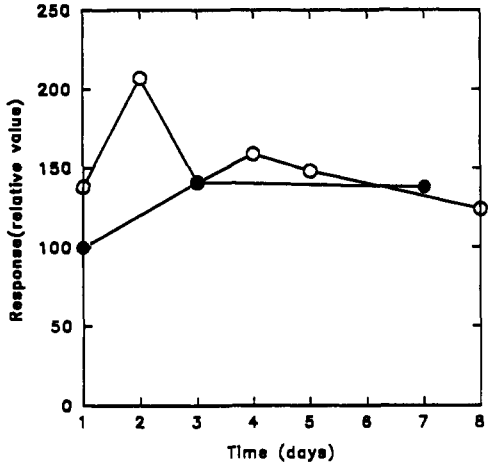

Figure 8. Storage stability of the immobilized microorganism (●) and operational stability of the microbial electrode (○).

The operational stability of the microbial electrode was examined using the third electrode (first use at the seventh day after preparation) measuring each day 10 samples; i.e., the electrode was continuously used during 5 h. As shown in Figure 8, no significant decrease in responses was observed even after 8 days. These results suggest that the microbial electrodes possess a good stability for at least 2 weeks after their preparation (7 days of storage + 8 days of operation).

The rancid flavor in milk is frequently determined by acid degree value (ADV). However, the accuracy of ADV in predicting sensory detection of rancidity is questionable (Duncan et al., 1991). There are definite requirements to an alternative method for ADV. The microbial sensor specific for short-chain fatty acids described here could meet these requirements of specificity, rapidity, and low cost. Testing with a large number of samples is in progress to determine the potential of this short-chain fatty acid determination system as an alternative to ADV. Additionally, the applicability of the electrode in a batch system will be tested, taking special care of the sensitivity and specificity of the sensor, which may change due to the internal dilution provided by the FIA system and to changes in the residence time of the sample at the sensor.

LITERATURE CITED

Deeth, H. C.; Fitz-Gerald, C. H.; Snow, A. J. A gas chromatographic method for the quantitative determination of free fatty acids in milk and milk products. *N. Z. J. Dairy Sci. Technol.* **1983**, *18*, 13–20.

de Jong, C.; Bading, H. T. Determination of free fatty acids in milk and cheese. Procedures for extraction, clean up, and capillary gas chromatographic analysis. *J. High Resolut. Chromatogr.* **1990**, *13*, 94–98.

Dole, V. P.; Meinertz, H. Microdetermination of long-chain fatty acids in plasma and tissues. *J. Biol. Chem.* **1960**, *235*, 2595–2599.

Duncan, S. E.; Christen, G. L.; Penfield, M. P. Rancid flavor of milk: Relationship of acid degree value, free fatty acids, and sensory perception. *J. Food Sci.* **1991**, *56*, 394–397.

Hosaka, K.; Kikuchi, T.; Mitsuhida, N.; Kawaguchi, A. A new colorimetric method for the determination of free fatty acids with acyl-CoA synthetase and acyl-CoA oxidase. *J. Biochem.* **1981**, *89*, 1799–1803.

Itaya, K.; Ui, M. Colorimetric determination of free fatty acids in biological fluids. *J. Lipid Res.* **1965**, *235*, 2595–2599.

Kuzdzal-Sovoie, S. Determination of free fatty acids in milk and milk products. *Int. Dairy Fed. Bull. Doc.* **1980**, *118*, 53–66.

Shimizu, S.; Yamada, H. Free fatty acids-colorimetric method. In *Methods in Enzymatic Analysis*; Bergmeyer, H. U., Ed.; VCH Verlagsgesellschaft: Weinheim, 1985; Vol. 8, p 19.

Sode, K.; Tamiya, E. Karube, I.; Kameda, Y. Sensor for free fatty acids based on acyl coenzyme-A synthetase and acyl coenzyme-A oxidase. *Anal. Chim. Acta* **1989**, *220*, 251–255.

Sugiura, M.; Oikawa, T.; Hirano, K.; Inukai, T. Purification, crystallization and properties of triacylglycerol lipase from *Pseudomonas fluorescens*. *Biochim. Biophys. Acta* **1977**, *488*, 353–358.

Wagner, G. Ph.D. Dissertation, Technischen Universität Berlin, 1991.

Walstra, P.; Jenness, R. *Dairy Chemistry and Physics*; Wiley: New York, 1984; p 336.

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