A MICROBIAL SENSOR FOR DETERMINATION OF SHORT-CHAIN FATTY ACIDS AND ITS APPLICATION TO RAW MILK SAMPLES

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ABSTRACT

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 (C4:0-C12:0), 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 sensor system was applied to determine free short-chain fatty acids in raw milk samples and the result was compared with gas chromatography (GC) and titrimetric method. The sensor response was linearly related to the total concentration of short-chain fatty acids obtained by GC ($n=10$, $r=0.92$) and to the titrimetric result ($n=10$, $r=0.78$). This result suggests that the present microbial sensor can selectively determine free short-chain fatty acids in raw milk samples and may be useful as a detection method of rancidity in milk.

INTRODUCTION

The rancid or off-flavor in milk and milk products is caused by the liberation of short-chain fatty acids (C4:0-C12:0) by milk or bacterial lipases (1, 2). 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 (3, 4). These methods show no specificity related to fatty acid chain length. As short-chain fatty acids represent about 5 to 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 is also possible (5, 6). This method enables the determination of each fatty acid separately, 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 and the procedure is generally complicated. Shimizu et al. (7) and Hosaka et al. (8) have proposed the enzymatic determination of free fatty acids. The method is based on monitoring the hydrogen peroxide formed by the two sequential reactions of the enzymes acyl-CoA synthetase and acyl-CoA oxidase. This method has some advantages in terms of selectivity and rapidity and a sensor system based on this method has already been developed (9). However, in the application of this method to detect the rancidity of dairy products there would be a problem in terms of specificity. The acyl-CoA oxidase commercially available has generally a high specificity for long-chain but only a low specificity for short-chain fatty acids, especially butyric acid (7, 8).

Wagner (10) found that the microorganism *Arthrobacter nicotiana* showed the 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 the 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.

MATERIALS AND METHODS

Chemicals. Casein (sodium salt, from bovine milk) and dialysis membrane (cellulose tubing that would retain 90-99% of a cytochrome c (M.W. 12,400) solution over a 20-h period) were obtained from Sigma

Chemicals Co. Polyvinyl alcohol (PVA; M.W. 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. *Arthrobacter nicotiana* (DSM No. 6707, Braunschweig, FRG) was cultured under aerobic conditions at 30℃ 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. A given amount of the cell mass was mixed with 0.1 M potassium phosphate buffer (pH 7.0) containing 5% PVA. The cell suspension was spread on a glass plate (5 cm x 2 cm) and dried at room temperature for 3 h and then overnight at 5℃.

Assembly of the microbial electrode. The microbial electrode consisted of the microorganism membrane, a teflon membrane, a dialysis membrane and an oxygen electrode (Schott Geräte GmbH). 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. The free fatty acids added to this solution were emulsified using ultrasonic treatment (10 min). This served as the sample solution. The emulsion thus formed was stable for one day.

FIA system. A schematic diagram of the flow system is shown in Fig.1. 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 and was 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. When the flow rate was set at 1.0 ml/min the dispersion ratio of the injected sample solution was about 8. The measurements were carried out at 30℃ 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.

Raw milk samples. Ten milk samples from three cows were used in

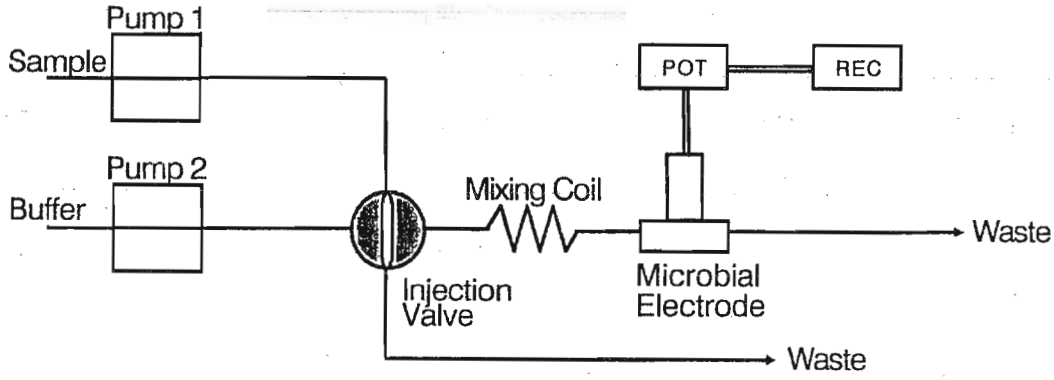
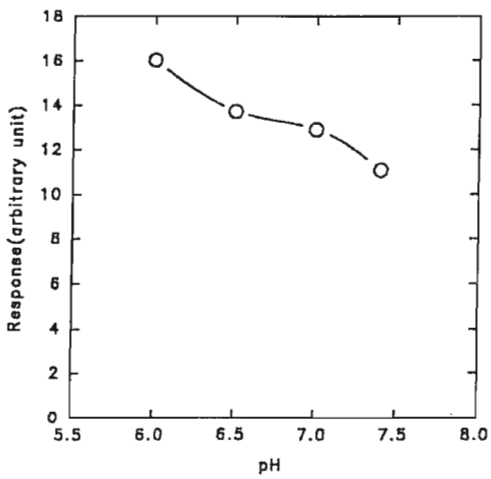


Fig. 1 *Schematic diagram of the flow injection analysis system. POT, potentiostat; REC, recorder.*

this experiment. Raw milks were frozen immediately after milking and stored in a deep-freeze at -20℃ before analyses. A special attention was paid to reduce the difference in the time intervals from milking to sampling at the three methods for each sample as little as possible.

Determination of free fatty acids in milk by gas chromatography (GC). A modification of the method described by de Jong and Bading (5). Five ml of milk sample with an internal standard (C7:0) was extracted with a mixture (16 ml) consisting of isopropanol/petroleumether/4 N sulphuric acid/ 2-butanol (44/45/1/10). The extract was applied to a disposable column with an weak anion exchanger (Supelco, Supelclean™ LC-NH_2 SPE Tube). Free fatty acids were eluted with ethylether (5 ml) containing 2% acetic acid after the fraction of neutral lipids was eluted with hexane. Following to evaporation of the solvent, the free fatty acids were derivatized with 1 ml of 15% boron trifluoride in ethylether/methanol (70/30) on heating at 100℃ for 5 min. The reaction was stopped by the addition of 1 ml of water and then the derivatized fatty acids were extracted with 0.5 ml of hexane. The derivatized fatty acids were analyzed on Perkin Elmer gas chromatograph model 8400, equipped with an FID. Thirty meters silica capillary column OV-225 was used for the separation of components. Carrier gas was nitrogen at a pressure of 85 kPa. The split ratio of the injected sample was regulated to 1:6. The temperature was programmed to rise from 55℃, with a 5 min hold at 30℃/min, to 190-℃, hold isothermal for 13 min at 30℃/min, to 195℃, for 6 min at 30℃/min, and then to 210℃. One μl of the sample was injected. Under these



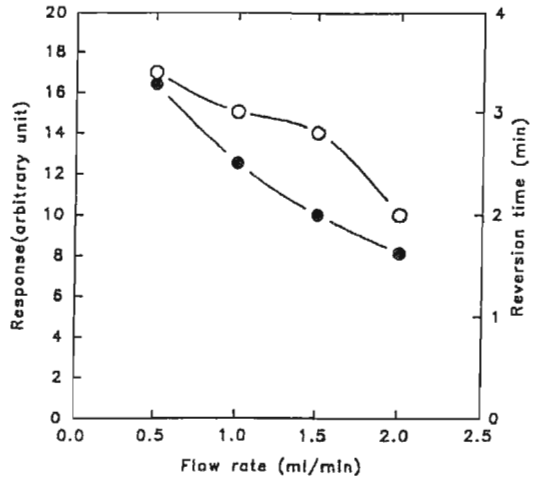


Fig. 2 *pH dependence of microbial electrode.*
Conditions are as follows: butyric acid concentration, 1.68 mM; flow rate, 1.0ml/min.
Fig. 3 *Effect of flow rate on response (●) and the time required for reversion to base line (○).*
Butyric acid concentration, 1.68 mM.

conditions, the following fatty acids could be determined for all samples; C4:0, C6:0, C8:0, C10:0, C12:0, C14:0, C16:0, C18:0, C18:1 and C18:2. The fatty acids from C4:0 to C12:0 were defined as a short-chain fatty acid.

Determination of free fatty acids in milk by titrimetry. The titrimetric determinations of free fatty acids were carried out by Lipo R-method described by Mathieu (11). An automatic burette Metrohm Dosimat E 535 was used for the titration and the end point was monitored at a wavelength of 620 nm with a photometer Metrohm 616, combined with a Metrohm Impulsomat E 614 to control the burret.

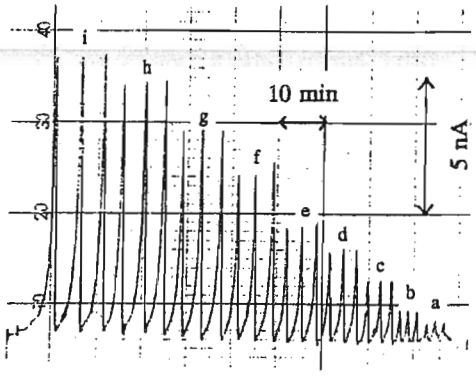
RESULTS AND DISCUSSION

The proposed sensor comprises a microorganism membrane, a protective membrane and an oxygen electrode. We have tried to apply the sensor to the analysis of milk. Milk is a complex mixture of many compounds, in which the content of casein is characteristically very high. Therefore, the first criterion for selecting a suitable protective membrane was that the

membrane pore size is small enough to prevent the casein molecule from reaching the microbial membrane. From this standpoint we have chosen a dialysis membrane that has a cut-off limit of about 12,000 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 (Fig.2). Although the initial responses gradually increased with a decrease in pH, the reaction buffer was kept at pH 7.0 in further experiments due to the higher stability of the microorganism at this pH. When the flow rate was varied over the range 0.5 to 2.0 ml/min, both the response and the time required for reversion to base line (90%) decreased with increasing flow rate (Fig.3). 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 (Fig.4) and the precision was better than 1.0% on five successive determinations. The sampling frequency was approximately 20 samples/h.

Optimum immobilization conditions. Figure 5 shows the effect of cell concentration in the PVA membrane on the system response. The



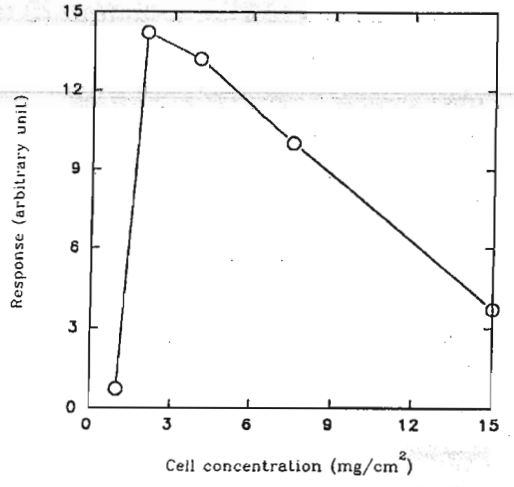


Fig. 4 *Typical response curve for butyric acid.*
a, 0.11 mM; b, 0.28 mM; c, 0.56 mM; d, 0.84 mM; e, 1.12 mM; f, 1.68 mM; g, 2.24 mM; h, 2.80 mM; i, 3.36 mM.

Fig. 5 *Effect of the amount of the cells of incorporated in the PVA membrane on the system response.*
Conditions are the same as in Fig. 2.

maximum response was observed with a cell concentration between 2 and 4 mg/cm^2. At a high concentration of microorganism, the response decreased drastically. Therefore, we selected 4 mg/cm^2 cell concentration for the future experiments.

Specificity of response for fatty acids. Sugiura et al. (12) and Sode et al. (9) prepared their standard solution by mixing free fatty acids and buffer containing bovine serum albumin as a model of human blood. In the present paper, we have prepared the solution by mixing fatty acids and buffer containing casein because it is a 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 (C4:0) to lauric acid (C12:0). These fatty acids are liberated by milk or bacterial lipases and are mainly responsible for the rancid flavor of dairy products (2). This result suggests that this sensor may be applicable to the detection of rancid flavor in dairy products.

Selectivity. In order to examine the selectivity of the microbial electrode, responses to the following compounds were determined; 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

Table I Comparison of responses for various fatty acids

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

Table II Recovery of added fatty acids into milk

fatty acids	added	Present	found	Recovery (%)
	concentration/mM			
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

(0.5%) and triolein (1%). Each concentration was adjusted to the usual level found in milk according to the literature (2). No response was observed for these compounds. The present method is based on the use of a microbial electrode covered with a dialysis membrane with a small pore size. In addition, the electrode is set in the continuous flow system, which means that the residence time of the injected sample is very short in the flow-through cell in which the microbial electrode is situated. Therefore, any specific compounds, with a high diffusion rate through the dialysis membrane and a high assimilation by the immobilized microorganism, would show a response in this system.

Application of the microbial electrode to raw milk samples. 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.

The relationship between the sensor response and the total concentration of short-chain fatty acids obtained by GC showed a good linearlity with a correlation coefficient of 0.916 (Fig.6) and the correlation was statistically

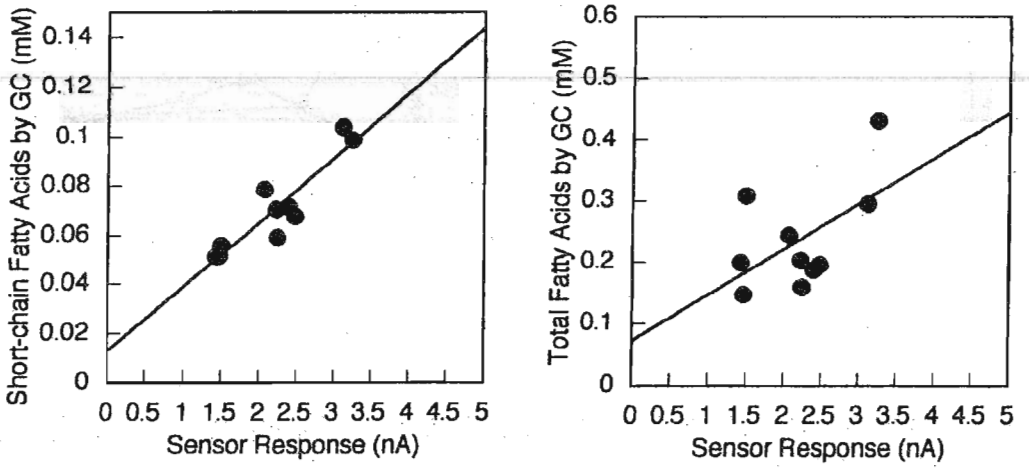


Fig. 6 *Relationship between the response obtained by the microbial sensor and total concentration of short-chain fatty acids (C4:0-C12:0) by gas chromatography (GC).*

Fig. 7 *Relationship between the response obtained by the microbial sensor and total concentration of free fatty acids by gas chromatography (GC).*

significant at the 0.1 level, while the correlation coefficient with the total concentration of all fatty acids was 0.559 (Fig.7). This result indicates that, for raw milk samples, the present system can detect short-chain fatty acids more selectively than long-chain fatty acids and the response obtained by the present sensor system was comparable to the total concentration of the short-chain fatty acids obtained by GC.

The response obtained by the present sensor was also linearly related to the titrimetric result with a correlation coefficient of 0.78. The conventional titrimetric method shows no specificity related to fatty acid chain length and reflects the total concentration of free fatty acids. Therefore the linear relationship between them would result from the fact that the concentration ratio of short-chain fatty acids to total fatty acid was approximately equivalent among all samples used. The titrimetric results were in the range 1.8-2.4 meq/l, hence the ratio of the maximum to the minimum was about 1.3. On the other hand, the ratio in the sensor responses was about 2.6 as shown in Fig.6. This means that the present sensor system can detect a difference

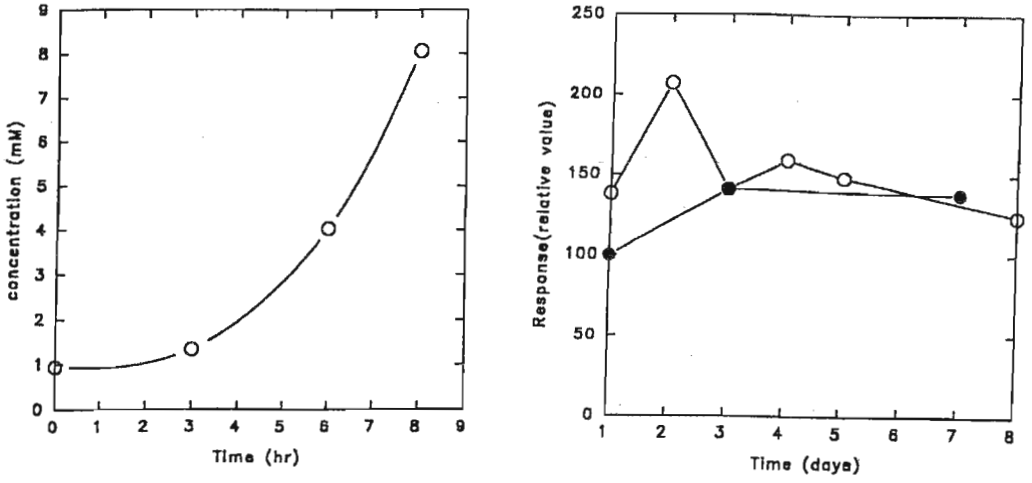


Fig. 8 *Relationship between storage time of agitated raw milk at 25℃ and sensor response.*
The concentration of short-chain fatty acids was calculated by using a calibration curve for butyric acid. The raw milk was diluted 5-fold and 10-fold with 0.1 M potassium phosphate buffer (pH 7.0) at 6 h and 8 h, respectively.

Fig. 9 *Storage stability of the immobilized microorganism (●) and operational stability of the microbial electrode (○).*

in the concentration of short-chain fatty acids more sensitively than the conventional titrimetric method.

Figure 8 shows the relationship between the storage time of agitated raw milk at 25℃ 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 and operational stability of the microbial electrode. Three microbial electrodes were prepared under the same conditions (Fig.9). The response of the first electrode was determined on the day of preparation. The remainder of the electrodes were stored in a nutritional medium containing butyric acid at 5℃ prior to use. With electrodes used on the third and seventh day following their preparation, no decrease in response was observed, on comparison with the response obtained on the first day. The operational stability of the microbial electrode was examined using the third electrode with which ten samples were continuously determined during 5-h operation. As shown in Fig.9, 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 two weeks after their preparation.

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 (13). There are clearly some demands to develop an alternative method to ADV. The microbial sensor specific for short-chain fatty acids described here would be a good candidate for these demands both in terms of specificity, rapidity and low cost.

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