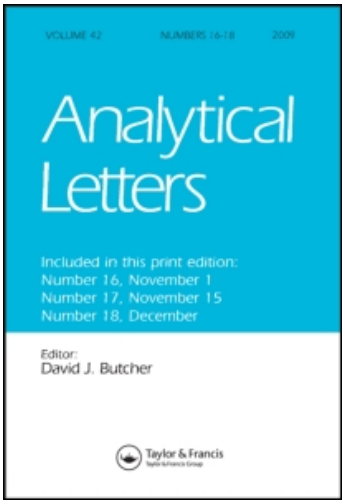

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Biosensors

SENSING THE LACTIC ACID IN PROBIOTIC YOGURTS USING AN L-LACTATE BIOSENSOR COUPLED WITH A MICRODIALYSIS FIBER INSERTED IN A FLOW ANALYSIS SYSTEM

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An amperometric biosensor for the determination of L-lactic acid in probiotic yogurts has been assembled using L-lactate dehydrogenase (EC 1.1.1.27, LDH) entrapped in 1% (v/v) neutralized Nafion® solution deposited on Variamine blue redox mediator modified screen-printed electrodes. The Variamine blue was previously covalently linked to oxidized single-walled carbon nanotubes and used for modifying screen-printed electrodes. The electrochemical cell, containing the L-lactate biosensor operating at an applied working potential of +200 mV vs. Ag|AgCl, was coupled with a microdialysis fiber and connected with a flow system, thus obtaining a microdialysis based sampling experimental set-up. Various analytical parameters, such as the cofactor concentration (2 mM, NAD^+), the flow rate (10.5 μL/min), the applied working potential (+200 mV vs. Ag|AgCl), the working buffer (50 mM phosphate buffer +0.1 M KCl), and pH (7.5), were optimized in batch amperometric experiments. The dynamic linear working range was comprised between $2 \cdot 10^{-4}$ and $1 \cdot 10^{-3}$ M. The proposed biosensor was challenged with real samples of yogurt, properly diluted in working buffer, and the performances of the L-lactate biosensor were compared with a commercially available kit for the determination of L-lactic acid in foodstuffs from R-Biopharm GmbH, Germany, showing a good agreement.

Keywords: Biosensor; L-lactic acid; Microdialysis fiber; Probiotic yogurts

1. INTRODUCTION

Produced naturally by lactic acid bacteria, D- and L-lactic acid can be found in many fermented milk products (yogurt, buttermilk, and cheese), in fermented vegetables (like sauerkraut or the Korean kimchi), or in cured meats and fish. L-lactic acid is added to foods and beverages (E270) where a tart flavor is desired, and is widely used as a non-volatile acidulant (Nikolaus and Strehlitzv 2008).

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Yogurt is derived from the Turkish word *Jugurt* describing any fermented food with acidic taste. Mainly, it is obtained from pasteurized or boiled milk soured by naturally occurring or lactic acid fermenting bacteria (FAO 1977), *i.e.*, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. Each of these organisms acidifies milk and produces specific yogurt flavors and aromas. Bourlioux and Pochart (1988) described yogurt as "coagulated milk" that resulted from the fermentation of lactic acid in milk by bacteria. The much defined characteristics (smooth texture, sour and pleasant flavor) of yogurt are attributed to the symbiotic fermentation involved in its manufacturing; nowadays, yogurt is one of the most widely distributed dairy products. Its composition is highly dependent on the type and source of milk and a range of seasonal factors. It is also significantly influenced by manufacturing conditions (such as temperature, duration, and equipment utilized) and by the presence of other ingredients such as powdered milk or condensed milk (Blanc 1986). Product quality and most of all consumer satisfaction are important for increasing the sales of various types of yogurt products (Barnes et al. 1991). The addition of probiotic bacteria during yogurt preparation is made not only because of the claimed health-promoting effects in the intestinal tract; other beneficial health effects are suggested, including enhancement of the immune system, reduction of lactose intolerance, and reduction of LDL cholesterol (so called "bad cholesterol") (Scheinbach 1999). Yogurt quality is difficult to standardize, because of its multitude of different forms, types, flavors, etc., and especially because of consumer preferences. For this reason, one of the key analytes in evaluating the quality of a yogurt is the amount of lactic acid. Mainly NAD^+ dependent lactate dehydrogenase, lactate oxidase or flavocytochrome b_2 amperometric based biosensors (Wagner and Guilbault 1994; Palmisano et al. 1995; Parra et al. 2006; Mizutani, Yabuki, and Hirata 1995; Palmisano et al. 1996; Collier, Janssen, and Hart 1996; Shu and Wu 2001; Collier, Lovejoy, and Hart 1998; Esti et al. 2004; Herrero et al. 2004; Palmisano et al. 2000; Kulys and Svirmickas 1980; Amine, Deni, and Kaufmann 1994; Smutok et al. 2005) are described in the literature as valuable sensing tools for lactate. Simon et al. (2002) described the immobilization of two genetically engineered forms of lactate dehydrogenase on poly(aniline)-poly(acrylate) and poly(aniline)-poly(vinylsulfonate) composite films, the produced NADH being oxidized at the glassy carbon modified electrode. This type of composite film-modified electrodes has the advantage as the modification is performed electrochemically. The deposited polymer, being in this case conductive, prevents the fouling of the electrode surface and also assists in the immobilization of enzymes within the film. Garjonyte, Melvydas, and Malinauskas (2006) proposed a carbon paste electrode modified with baker's yeast *Saccharomyces cerevisiae*, as a natural source of flavocytochrome b_2. Working at a potential close to 0 V vs. Ag/AgCl, the simple preparation and the stability of the prepared pastes (minimum 1 month) partially compensated for some of the disadvantages like the limited linear working range (0.03 to 1 mM), the high noise level and the pronounced sensitivity to ascorbic acid. A bienzyme amperometric graphite-Teflon composite biosensor, in which the lactate oxidase and the horseradish peroxidase were incorporated together with the redox mediator ferrocene, was reported by Serra et al. (1999) as a lactate biosensor for wine and yogurt, this bienzyme sensor being fabricated by simple physical inclusion of the two enzymes and the mediator in the bulk of the graphite-Teflon matrix. Recovery studies were

conducted and mean recoveries were assessed to be $102 \pm 5\%$ for red wine, and $100 \pm 7\%$ for yogurt. Sol–gel encapsulation of lactate dehydrogenase and NAD^+ represented a different approach in developing a disposable optical sensor for L-lactate. Although problems such as diminished enzymatic activity and/or leaching of enzyme from the sol–gel matrix occurred, the sol–gel process was sufficiently mild to permit retention of enzymatic activity. As reported by the authors, the sensor had a linear dynamic range over the normal physiological L-lactate level and showed a long-term storage stability of at least 3 weeks (Li et al. 2002). In this paper we describe an amperometric biosensor for the detection of L-lactic acid in probiotic yogurts, based on the use of Variamine blue-carbon nanotube modified screen-printed electrodes and L-lactate dehydrogenase (EC 1.1.1.27) as biological sensing element. The L-lactate biosensor was coupled with a microdialysis fiber which was inserted in a flow system, thus obtaining a microdialysis based sampling experimental set-up. The proposed biosensor was challenged with real samples of yogurt, properly diluted in working buffer, and the performances of the L-lactate biosensor were compared with a commercially available kit for the determination of L-lactic acid in foodstuffs from R-Biopharm GmbH, Germany.

2. EXPERIMENTAL

2.1. Reagents and Instrumentation

All chemicals from commercial sources were of analytical grade. Nafion® 5% (v/v) solution, L-lactate dehydrogenase (EC 1.1.1.27, L-LDH, 142 IU mg^{-1}), β-NAD^+ (>95%, HPLC) and NADH (>97%, HPLC) were purchased from Fluka Chemie. Potassium dihydrogen phosphate and potassium chloride were from Carlo Erba Reagenti; dibasic potassium phosphate was supplied by Riedel-de Haen and L-(+)-lactic acid was from Sigma, St. Louis, USA.

Measurements were carried out using a flow analysis (FA) system consisting of a peristaltic pump (Gilson model Minipuls 3, France) equipped with Tygon tubes, a homemade thin-layer flow through cell ($d_{int} = 15$ mm), and a microdialysis fiber. Microdialysis hollow fibers (Filtral AN69) were made of polyacrylonitrile metallyl-sulfonate (Hospal Industrie, Meyzieu, France) with about a 30 kDa cut-off, 200 μm of diameter and 2 cm of length. A 641 VA Detector (Metrohm, Switzerland), connected to an X–t recorder (L250E-Linseis, Germany) was used to record the analytical response.

2.2. Screen-Printed Electrodes (SPEs) Modified with Variamine Blue (VB)

Screen-printed electrodes were fabricated using a high performance multipurpose screen printer DEK 245, DEK, UK. The substrate was Autostat HT5 (polyester, 0.175 mm) from Autotype, Italy, and the electrodes were printed using the classic configuration, i.e., a working (WE), a counter (CE), and a reference electrode (RE). The reference electrode was screen-printed using Elettrodag 477 SS RFU, while for the CE and the WE a graphite based Elettrodag 423 SS RFU ink was utilized. The Elettrodag 6010 SS ink was used as insulator in the final printing step;

all inks were bought from Acheson Colloiden Italiana, Italy. Following the silver layer screen printing 10 min at 100°C were needed, while for the graphite based layers, 120°C and 10 min were necessary to promote the adhesion to the polyester substrate. For the insulator layer, 20 min at 70°C were the conditions applied.

Screen-printed electrodes modified with carbon nanotubes and Variamine blue (4-amino-4′-methoxydiphenylamine) were obtained as described in (Radoi et al. 2008). Briefly single-walled oxidized carbon nanotubes (Tamburri et al. 2005) were activated with N,N′-dicyclohexylcarbodiimide and N-hydroxysuccinimide, in N,N-dimethylformamide, in order to covalently link the redox mediator Variamine blue. Finally, the derivatized carbon nanotubes, i.e., SWCNTs-CO-VB, were deposited onto the working electrode so that it could subsequently be used as a NADH sensing probe.

2.3. L-Lactate Biosensor

Briefly, 2 μL of an enzymatic solution containing 2 IU of L-LDH/μL prepared in 1% (v/v) neutralized Nafion® solution were deposited onto the modified electrodes and allowed to dry. The L-lactate biosensor, when not used, was stored in working buffer (50 mM phosphate buffer +0.1 M KCl, pH 7.5) at 4°C.

2.4. Samples and Sample Preparation

Commercially available probiotic yogurt samples were bought from local markets. Samples were centrifuged 15 min at 6000 rpm (Beckman centrifuge-model J2–21), and the supernatant was carefully collected and diluted (1/100, 1/50 and 1/10 v/v) prior analysis. All the samples were also assayed using a commercially available kit for the determination of L-lactic acid in foodstuffs from R-Biopharm GmbH, Germany.

3. RESULTS AND DISCUSSION

The enzymatic determination of L-lactic acid is based on the following reaction scheme:

$$\text{L} - \text{lactic acid} + \text{NAD}^+ \overset{\text{L-LDH}}{\longleftrightarrow} \text{Pyruvic acid} + \text{NADH} + \text{H}^+ \quad (1)$$

When the L-lactate dehydrogenase is coupled with an NADH amperometric sensing electrode, it is easy to monitor and quantify the amount of L-lactic acid in samples, based on the fact that the NADH is oxidized at the electrode, producing an oxidation current directly proportional with the L-lactic acid concentration:

$$NADH \rightarrow NAD^+ + \text{H}^+ + 2\text{e}^- \quad (2)$$

However, direct oxidation of NADH at bare electrodes occurs at overpotentials (up to 1.0 V), is highly irreversible, proceeds with coupled side reactions, and inevitably poisons the electrode surface (Blaedel and Jenkins 1975; Samec and Elving 1983; Moiroux and Elving 1978). Consequently, considerable efforts have been done for

the identification of new electrode materials and redox mediators capable to reduce of the oxidation overpotential and to improve the electron transfer rate. Screen-printed electrodes modified with Variamine blue (Radoi et al. 2008) showed good catalytic activity towards the NADH oxidation, since they can oxidize the NADH molecule at convenient applied potentials. If the formed NADH is immediately oxidized at the Variamine blue modified electrodes, a net driving force for lactate oxidation will be established and an oxidation current will be produced, reflecting the concentration of lactate in samples.

Microdialysis is a reliable technique adequate for continuous sampling procedures, the perfused semipermeable membrane acting as a barrier to large interfering molecules (proteins, enzymes, dextrins, etc.). An appropriate molecular weight cut-off of the membrane improves the selectivity, avoiding time consuming sample preparation and clean-up procedures. Thus, the need for preparative and clean-up steps is minimized, reducing the total time of analysis with great benefits for a continuous monitoring process. Because yogurt is a very complex matrix and in order to avoid, as before described, preliminary treatments like protein precipitation, etc., a microdialysis sampling protocol (Figure 1) was used, since this approach has proved to be a powerful technique for food analysis (Marrazza, Cagnini, and Mascini 1994; Mannino, Cosio, and Zimei 1996).

Batch amperometric investigations were conducted in order to establish the optimum working applied potential, as well as the best concentration of NAD^+. The investigations were conducted using phosphate buffer (50 mM, pH 7.5) + KCl (0.1 M), as the working buffer. Applying an optimized working potential of +200 mV (*vs.* Ag|AgCl) and using a previously optimized 2 mM NAD^+ concentration, the L-lactate biosensor was inserted into a flow analysis system coupled with a microdialysis fiber. The mobile phase was represented by the working buffer enriched with 2 mM NAD^+ and the optimum flow rate was 10.5 μL/min. The microdialysis fiber was connected (In/Out) to the main stream of the mobile phase by a 4 way injection valve from Rheodyne, USA. Once the experimental set-up was in place, a calibration curve was obtained using kwon concentrations of standard solutions of L-lactate. As it can be observed from Figure 2, the dynamic linear working range (LWR) is approximately wide one order of magnitude, being comprised between $2 \cdot 10^{-4}$ and $1 \cdot 10^{-3}$ M. Compared with the linear working range obtained in batch amperometric experiments, which is wide about two orders of magnitude

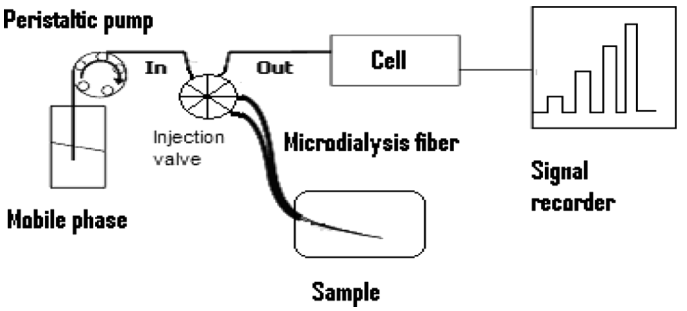

Figure 1. Experimental set-up of the flow analysis system equipped with a microdialysis hollow fiber.

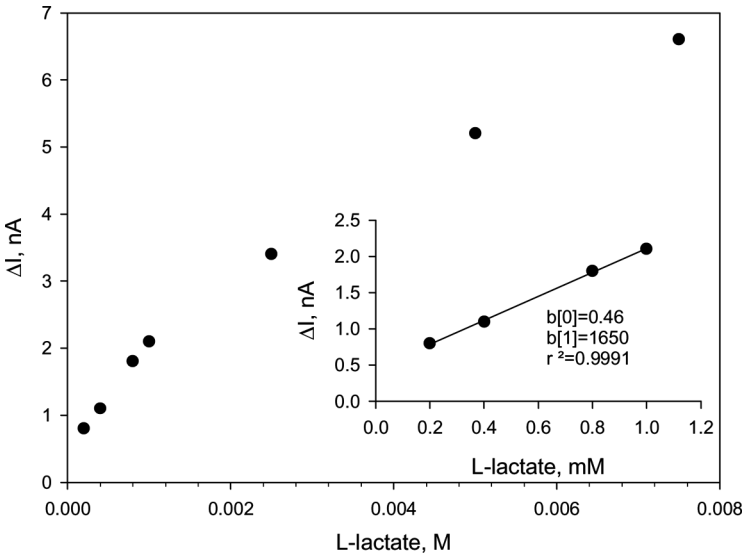

Figure 2. Calibration curve obtained using the experimental set-up described in Figure 1; +200 mV *vs.* Ag|AgCl; mobile phase: phosphate buffer (50 mM, pH 7.5) + KCl (0.1 M) + 2 mM NAD^+; flow rate: 10.5 μL/min.

($5 \cdot 10^{-6}$ and $4.5 \cdot 10^{-4}$ M), it is evident that both the flow rate and the presence of the microdialysis fiber greatly influences the linear working range. This drastic decrease in linearity was mainly due to the membrane properties, hydrodynamic variables, and diffusivity of the analyte, parameters that are known to determine the permeability factor, which governs the relative recovery (RR) in microdialysis sampling (Bungay, Morrison, and Dedrick 1990):

$$\mathrm{RR} = 100\{1 - 1/\exp[Q_\mathrm{p}(R_\mathrm{m} + R_\mathrm{e} + R_\mathrm{d})^{-1}]\} \tag{3}$$

R_m – diffusional mass transport resistance through the membrane;
R_e – diffusional mass transport resistance through the external media;
R_d – diffusional mass transport resistance through the dialysate;
Q_p – flow rate of the perfusate;
$1/(R_\mathrm{m} + R_\mathrm{e} + R_\mathrm{d})$ – permeability factor.

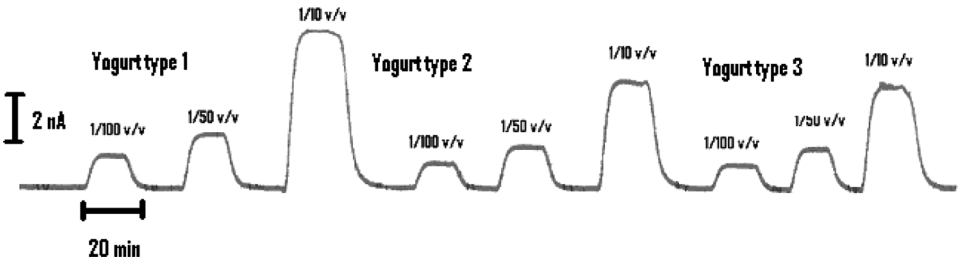

Figure 3. Flow analysis amperometric recordings of real samples of yogurt centrifuged (15 min at 6000 rpm) and diluted (1/100, 1/50, and 1/10 v/v, in working buffer).

Table 1. Content of L-lactic acid in three different types of commercially available yogurts

	Biosensor							Spectrophotometric assay		
	ΔI, nA				L-lactic acid, %			L-lactic acid, %		
Dilution, v/v	Yogurt type 1	Yogurt type 2	Yogurt type 3	Equation	Yogurt type 1	Yogurt type 2	Yogurt type 3	Yogurt type 1	Yogurt type 2	Yogurt type 3
1/100	1.7	1.3	1.25	$y = 0.46 + 1650x$	0.68 [RE: +3%]	0.46 [RE: −6%]	0.43 [RE: −10%]	0.66 [−]	0.49 [−]	0.48 [−]

Once that the LWR was assessed, diluted (1/100, 1/50 and 1/10 v/v, in order to fit the linear working range) samples of yogurt were assayed using the flow analysis system equipped with the microdialysis fiber, and the analytical signal was recorded (Figure 3). The results obtained using the L-lactate biosensor were compared (Table 1) with the data obtained using the kit for the determination of L-lactic acid in foodstuffs from R-Biopharm GmbH, Germany, obtaining a good degree of correlation.

4. CONCLUSIONS

In this paper, we described the analytical properties of an L-lactate biosensor utilized as a sensing element for L-lactic acid determination in probiotic yogurts.

The experimental set-up consisted in a flow analysis system equipped with a microdialysis hollow fiber connected to the electrochemical cell, containing the L-lactate biosensor.

The benefits arisen from using microdialysis (continuous sampling and monitoring, reducing potential interfering molecules, reduced preparative procedures, etc.) are sometimes counterbalanced, like in this case, by to a certain extent influenced analytical parameters, like the linear working range, which is an important issue to deal with in the field of biosensors.

The proposed biosensor was challenged with real samples of probiotic yogurt, properly diluted in working buffer, and the performances of the amperometric L-lactate biosensor were confronted with the results obtained from spectrophotometric readings, using a commercially available kit for the determination of L-lactic acid in foodstuffs, showing a good correlation. Moreover, the proposed system is fast and easy to use, permitting the analysis of a great number of samples in sequence and in a short period of time.

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