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RESEARCH ARTICLE

Amperometric bienzymatic biosensor in flow injection analysis system for determination of aspartame in foods

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Abstract

An amperometric bienzymatic biosensor was developed for the determination of aspartame in a flow injection analysis (FIA) system, consisting of two enzyme reactor columns packed with immobilized α-chymotrypsin (CHY) and alcohol oxidase (AOX) beads and a hydrogen peroxide electrode, connected in series. The CHY and AOX were separately immobilized on glutaraldehyde (GA)-activated beads through covalent bonding. The biosensor fabrication and operational conditions were optimized. The optimal fabrication conditions were: 2% GA with 120 min activation time; and 250 U/mL CHY and 100 U/mL AOX, with 180 min enzyme immobilization time. The optimal operational conditions were a flow rate of 0.5 mL/min and pH 8.0 at room temperature. The developed biosensor showed linearity over the aspartame concentration range 0.01–1.2 mM, with a detection limit of 0.005 mM. The developed biosensor was satisfactorily applied for detecting aspartame in beverage samples without any excessive pretreatments.

Keywords Biosensor · Aspartame · Flow injection analysis · Chymotrypsin · Alcohol oxidase

Introduction

Aspartame (L-aspartyl-L-phenylalanine methyl ester) is broadly used as a sweetening agent and/or a flavor enhancer in numerous food, beverage, and pharmaceutical products in more than 90 countries (Choudhary and Pretorius, 2017; Magnuson et al., 2007). Aspartame is an artificial intense sweetener (approximately 180–200 times that of sucrose) which has a sugar-like taste with no bitter or metallic aftertaste (Choudhary and Pretorius, 2017; O'Donnell, 2012). In the gastrointestinal tract, aspartame is hydrolyzed to phenylalanine, aspartic acid and methanol with percentages of 50%, 40% and 10%, respectively (Czarnecka et al., 2021). The high percentage of phenylalanine generated from aspartame is hazardous to individuals with phenylketonuria who cannot convert phenylalanine to tyrosine due to the lack of phenylalanine hydroxylase, leading to the accumulation of phenylalanine in the brain and blood that can result in many adverse health effects (Czarnecka et al., 2021). Patients with phenylketonuria are recommended to avoid consuming aspartame to restrict the intake of phenylalanine (Czarnecka et al., 2021; O'Donnell, 2012). Therefore, it is crucial to develop a method for the accurate and sensitive detection of aspartame in food products.

The methods used for aspartame determination include high performance liquid chromatography (HPLC) (Trandafir et al., 2009), spectrophotometry (Fatibello-Filho et al., 1999), and HPLC-mass spectrometry (Lim et al., 2013). These methods are time-consuming and require highly skilled personnel and excessive sample pretreatment. Biosensors are analytical devices that combine a biological recognition element with a transducer, which can generate a measurable signal that is proportional to the concentration of the target analyte (McGrath et al., 2012). Biosensors provide several benefits over conventional methods, including high selectivity, potential for miniaturization and automation, and rapid analysis and monitoring of analytes.

Several biosensors have been developed for aspartame detection. A microbial amperometric sensor for aspartame has been described based on *Bacillus subtilis*, which measures the respiration rate change (Renneberg et al., 1985). However, this sensor was slightly interfered with by glucose

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and the amino acid components of aspartame (L-aspartate and L-phenylalanine). Other sensors that have been reported include: a potentiometric sensor for detecting aspartame based on co-immobilization of carboxypeptidase A and L-aspartase on an ammonia electrode (Fatibello-Filho et al., 1988); and a flow injection analysis (FIA) biosensor system for determination of aspartame using peptidase, aspartate aminotransferase and glutamate oxidase (Male et al., 1991). These sensors have a major drawback caused by interference from L-aspartate. In addition, a tri-enzyme biosensor system is associated with considerably increased cost. An amperometric enzyme electrode for the detection of aspartame has been proposed using α-chymotrypsin (CHY) in solution and immobilized alcohol oxidase (AOX) on an oxygen electrode (Smith et al., 1989). In addition, amperometric bi-enzyme sensors for aspartame have been described based on immobilizing CHY and AOX on a membrane and mounting on an oxygen or hydrogen peroxide electrode (Chou, 1996; Compagnone et al., 1997), as well as bi-enzyme biosensors for detecting aspartame based on carboxyl esterase and alcohol oxidase (Kirgöz et al., 2006; Radulescu et al., 2014).

In an FIA system, the enzymatic step can be separated from the detection step by immobilizing the enzymes on an appropriate support and packing into a compact column reactor, while the formed product/coproduct can be subsequently measured using a suitable detection device. The utilization of immobilized enzymes packed into small column reactors in the FIA system has several merits, such as strict repetition, selectivity, and economy (Hansen, 1994). The highly immobilized enzyme concentration obtained within a small volume and the considerably more intense mass transfer in a packed reactor compared to a diffusion-controlled membrane result in rapid and extensive substrate conversion (Hansen, 1994). Therefore, in the present work, an amperometric bi-enzyme biosensor was developed for detection of aspartame in an FIA system based on covalently immobilizing α-chymotrypsin (CHY) and alcohol oxidase (AOX) on glutaraldehyde-activated beads and packing into enzyme reactors. CHY converts aspartame to L-aspartyl-L-phenylalanine and methanol. Subsequently AOX converts methanol to formaldehyde and hydrogen peroxide. The generated hydrogen peroxide can be detected amperometrically. The fabrication parameters (GA concentration, activation time, the amount of enzyme, and enzyme immobilization time) and operating parameters (flow rate, pH, and temperature) were optimized. In addition, the operational stability of the developed biosensor was examined. Finally, the proposed biosensor was applied for detection of aspartame in real food samples.

Materials and methods

Materials and chemicals

The beverage samples were purchased from a local supermarket (BKK, Thailand). α-Chymotrypsin from bovine pancreas (EC 3.4.21.1), alcohol oxidase from *Pichia pastoris* (EC 1.1.3.13), glutaraldehyde (25% aqueous solution) and tris(hydroxymethyl) aminomethane were purchased from Sigma-Aldrich, USA. Aspartame and concentrated hydrochloric acid were purchased from Supelco, USA. Sodium di-hydrogen phosphate and di-sodium hydrogen phosphate were purchased from Kemaus, Australia. All chemicals used in this study were of analytical or HPLC grade.

Measurement of sensor response

The flow injection analysis system (Fig. 1A) consisted of a carrier reservoir, a peristaltic pump (Miniplus 3; Gilson; France), a rheodyne injector (model 7225i; Cotati; USA), two enzyme reactor columns (reactor I: immobilized CHY column and reactor II: immobilized AOX column) and an electrochemical cross flow cell installed with a dual platinum disk working electrode (3 mm diameter), a Ag/AgCl (3 M NaCl) reference electrode and a stainless steel tube counter electrode (BAS; Japan). The working electrode was poised at + 700 mV versus a Ag/AgCl (3 M NaCl) reference electrode using a potentiostat (Metrohm Autolab; the Netherlands) to detect the hydrogen peroxide produced from the enzymatic reaction.

The peristaltic pump delivered a 0.1 M phosphate buffer solution (PBS) at pH 8.0 as a carrier solution to the enzyme reactors and flow cell equipped with the three-electrode system at room temperature at a flow rate of 0.5 mL/min, unless otherwise noted. The response signal was recorded using the Nova 2.1 software (Metrohm Autolab; the Netherlands). After the baseline signals had been established, the sample was injected through a 100 µL loop and delivered to enzyme reactors I and II, respectively. The obtained response signal was recorded. All measurements were conducted in triplicate.

Enzyme immobilization method

The present study used supports (based on spherical cellulose beads having a primary amine as an active group) for immobilization of the enzymes. CHY and AOX were immobilized on the beads separately using a covalent binding method. First, the beads were incubated in 0.1 M PBS (pH 7.5) overnight at 4 °C, followed by washing three times with the same buffer. Then, the beads were activated by

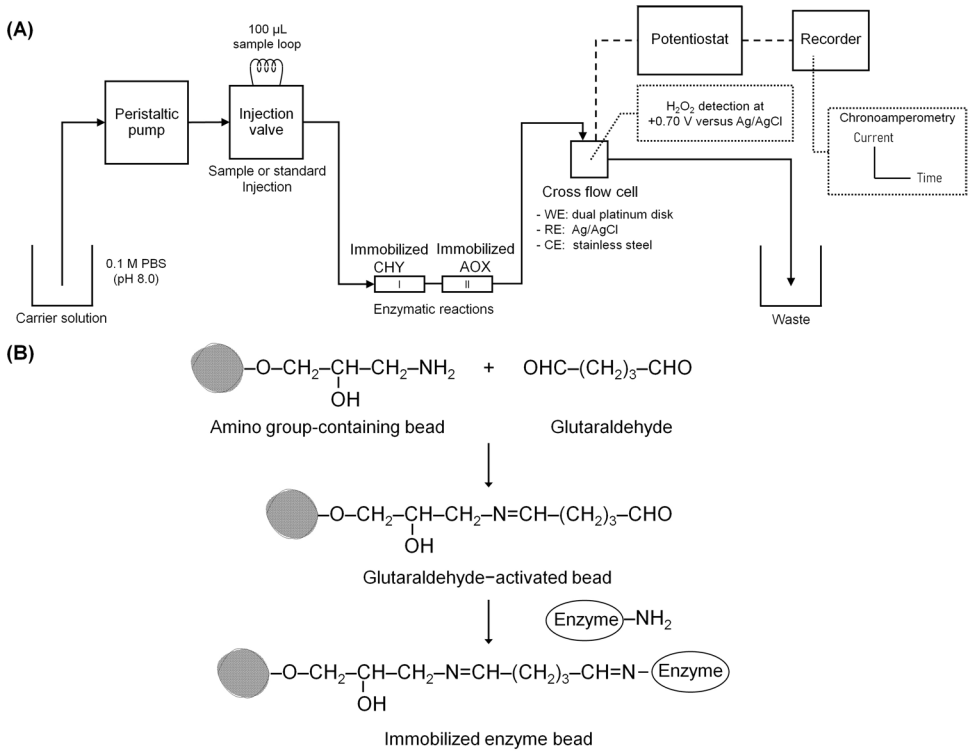

Fig. 1 (**A**) Schematic diagram of flow injection analysis system based on immobilized CHY and AOX, and amperometric detection. (**B**) Schematic representations of enzyme immobilization on activated bead

adding 2% GA (12 mL) into the beads (6 g) and slowly rotating using a roller mixer (SRT6D; Stuart; UK) for 120 min at room temperature. After activation with the GA, the treated beads were washed three times with 0.1 M PBS (pH 7.5). Then, 250 U/mL of CHY and 100 U/mL of AOX enzyme solutions (each 6 mL) in 0.1 M PBS (pH 7.5) were separately prepared and added to each 3 g of the activated beads and both mixtures were slowly rotated using the roller mixer for 180 min at 4 °C. Then, the obtained beads with each immobilized enzyme were washed thoroughly with 0.1 M PBS (pH 7.5) at 4 °C and stored in the same buffer at 4 °C. The immobilized CHY and AOX beads were packed separately into borosilicate columns (3 mm inner diameter and 25 mm length) for use as enzyme reactor I (immobilized CHY reactor) and enzyme reactor II (immobilized AOX reactor), respectively, in our FIA system.

Optimization of fabrication conditions for biosensor

Effects of glutaraldehyde concentration and activation time on sensor response

Amino group-containing beads were activated with different GA concentrations (1, 2, 3 or 4%) and activation times (5, 60, 120, 180 or 240 min). The obtained activated beads were used to separately immobilize CHY (250 U/mL) and AOX (100 U/mL) using an immobilization time of 180 min. The responses of the resulting biosensor to 0.3 mM aspartame were detected at an applied potential of + 700 mV versus Ag/AgCl (3 M NaCl) using 0.1 M PBS (pH 8.0) and a flow rate of 0.5 mL/min at room temperature.

Effects of amounts of enzymes and immobilization time on sensor response

To investigate the effect of the amounts of enzymes, the beads activated using the optimal GA concentration and activation time obtained from the above experiments were used to separate immobilized CHY and AOX using different amounts of CHY (50, 250 or 500 U/mL) and AOX (50 or 100 U/mL) with an immobilization time of 180 min. The current responses observed with the prepared biosensor to 0.3 and 0.5 mM aspartame were detected at an applied potential of + 700 mV versus Ag/AgCl (3 M NaCl) using 0.1 M PBS (pH 8.0) and a flow rate of 0.5 mL/min at room temperature.

To determine the optimal enzyme immobilization time, the beads activated using the optimal GA concentration and activation time were used to immobilize CHY and AOX using the determined amounts of enzymes from above with different enzyme immobilization times (5, 60, 120, 180 or 240 min). The responses of the fabricated biosensor to 0.3 and 0.5 mM aspartame were detected at an applied potential of + 700 mV versus Ag/AgCl (3 M NaCl) using 0.1 M PBS (pH 8.0) and a flow rate of 0.5 mL/min at room temperature.

Optimization of operational conditions for biosensor

Effect of flow rate on sensor response

The aspartame biosensors based on immobilized CHY and AOX were prepared using the optimal fabrication conditions obtained from the above experiments. The sensor responses to 0.3 and 0.5 mM aspartame were measured at room temperature using 0.1 M PBS (pH 8.0) as a carrier solution with varying flow rates (0.2, 0.3, 0.4, 0.5 or 0.6 mL/min).

Effect of pH on sensor response

The bienzymatic biosensors for aspartame were prepared using the optimal fabrication conditions determined above. The sensor responses to 0.3 and 0.5 mM aspartame were determined using the optimal flow rate obtained from the above experiment at room temperature using 0.1 M PBS with varying pH values of 6.5, 7.0, 7.5 or 8.0 and 0.1 M Tris–HCl buffer solution (pH 8.5 or 9.0) as carrier solutions.

Effect of temperature on sensor response

The bienzymatic biosensors for aspartame were prepared using the optimal fabrication conditions examined above. The sensor responses to 0.3 and 0.5 mM aspartame were measured using the optimal pH and flow rate examined above at different temperatures (15, 20, 25, 30, 35, 40, 45 or 50 °C).

Analytical performance of the developed bienzymatic FIA biosensor for aspartame

The current responses were determined under the optimal operating conditions of the aspartame biosensor based on immobilized CHY and AOX fabricated using the optimal preparation conditions. Each 100 µL of the standard aspartame solution (0.005–3.0 mM) was injected into the developed FIA biosensor system and the obtained current responses were recorded. A calibration curve was plotted between the current response and aspartame concentration.

Operational stability of biosensor

The bienzymatic biosensor for aspartame was prepared using the proper fabrication conditions determined from the above experiments. The operational stability of the biosensor was examined based on 60 successive measurements of 0.1 mM aspartame under the above obtained optimal operating conditions for a period of 10 h.

Determination of aspartame in real food samples

The developed bienzymatic biosensor was applied for determination of aspartame in real food samples, consisting of commercial soft drink and 25% fruit juices (orange juice with nata de coco, and peach and white grape juice with yoghurt and nata de coco). The soft drinks were passed through a 0.45 µm syringe filter, while the 25% fruit juice samples were centrifuged at 10,000×*g* for 20 min before passing through a 0.45 µm syringe filter. Then, the filtered samples (each 100 µL) were injected into the developed bienzymatic FIA biosensor system for aspartame, and the obtained current responses were recorded and the aspartame concentration was calculated using the calibration curve. The aspartame concentration in these samples was also examined using the HPLC method based on British Adopted European Standard BS EN 12856 (1999). The samples were analyzed in triplicate for both techniques.

Statistical analysis

The measurement of sensor response and the determination of aspartame concentration in the beverage samples using different techniques were conducted in triplicate and the results were presented as the mean ± standard deviation (SD). Data were analyzed using analysis of variance (ANOVA) and Tukey's test, unless otherwise stated. A *t* test was used to determine differences between the means of sensor response of the developed aspartame biosensor fabricated using two different amounts of AOX at a given amount of CHY and between means of the aspartame content in the beverage samples determined by the two different techniques. Statistically significant differences were evaluated at the 95% confidence level. Data analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 26 software (IBM; USA).

Results and discussion

In this study, a bienzymatic FIA biosensor was developed for detection of aspartame on the basis of separate covalent immobilization of α-chymotrypsin (CHY) and alcohol oxidase (AOX) on an activated support. The technique was based on a treatment of amino group-containing beads with glutaraldehyde (GA) as the activating agent to achieve a carbonyl derivative, which was bound covalently to the enzyme (Singh et al., 2017). The amino groups of the support and an aldehyde group of GA interacted in a first Schiff-base reaction (Fig. 1B). Then, the second Schiff-base reaction

occurred between the second aldehyde group of GA and an amino group of the enzyme (Sassolas et al., 2012). CHY catalyzed the cleavage of aspartame as shown in Eq. 1 to produce methanol that was converted by AOX to formaldehyde and hydrogen peroxide, as shown in Eq. 2 (Peña et al., 2004). The subsequent amperometric detection of hydrogen peroxide was performed at an applied voltage of + 700 mV versus Ag/AgCl.

$$\text{Aspartame} + H_2O \xrightarrow{\alpha\text{-chymotrypsin}} \text{L-aspartyl-L-phenylalanine} + \text{methanol} \quad (1)$$

$$\text{Methanol} + O_2 \xrightarrow{\text{alcohol oxidase}} \text{formaldehyde} + H_2O_2 \quad (2)$$

To determine the optimal conditions for the fabrication and operation of the proposed aspartame biosensor based on immobilized CHY and AOX in the FIA system, the fabrication parameters were optimized, consisting of GA concentration, activation time, the amounts of enzymes (CHY and AOX), and enzyme immobilization time; the operational parameters were also optimized, consisting of flow rate, pH, and temperature.

Optimization of fabrication conditions for biosensor

Effects of glutaraldehyde concentration and activation time on sensor response

Figure 2 shows the effect of glutaraldehyde concentration on the responses of the developed bienzymatic FIA biosensor for aspartame. As the concentration of GA increased from 1 to 2%, the sensor response to 0.3 mM aspartame increased significantly ($p < 0.05$). With a further increase in GA concentration from 2 to 4%, the sensor response significantly decreased. The highest sensor response was achieved with 2% GA. Similar results were reported by Singh et al. (2017) and Nwagu et al. (2021). At a low concentration, there may not have been sufficient GA for enzyme immobilization and hence a smaller amount of enzyme was immobilized, resulting in a low sensor response. However, a higher concentration of GA may cause enzyme denaturation and the loss of enzyme flexibility, which is essential for substrate binding, leading to a decreased sensor response (Yılmaz et al., 2012). In addition, the excessive concentration of GA may cause multipoint binding of enzymes with the support, resulting in spatial structural obstacles to inactivate the enzyme, while more enzymes bound to the support may cause the change in the spatial structure of the active site of the enzyme, resulting in a decrease in enzyme activity (Chen et al., 2013).

The influence of GA activation time on sensor response was also investigated. The inset of Fig. 2 shows the effect of GA activation time on sensor response. With increasing GA activation time from 5 to 120 min, the sensor response significantly increased. However, when the activation time was extended from 120 to 240 min, there was little change in the sensor response. A similar tendency was observed by Nwagu et al. (2021).

Therefore, 2% GA and 120 min activation time were selected as the optimal GA concentration and activation time for fabricating the aspartame biosensor in the present study.

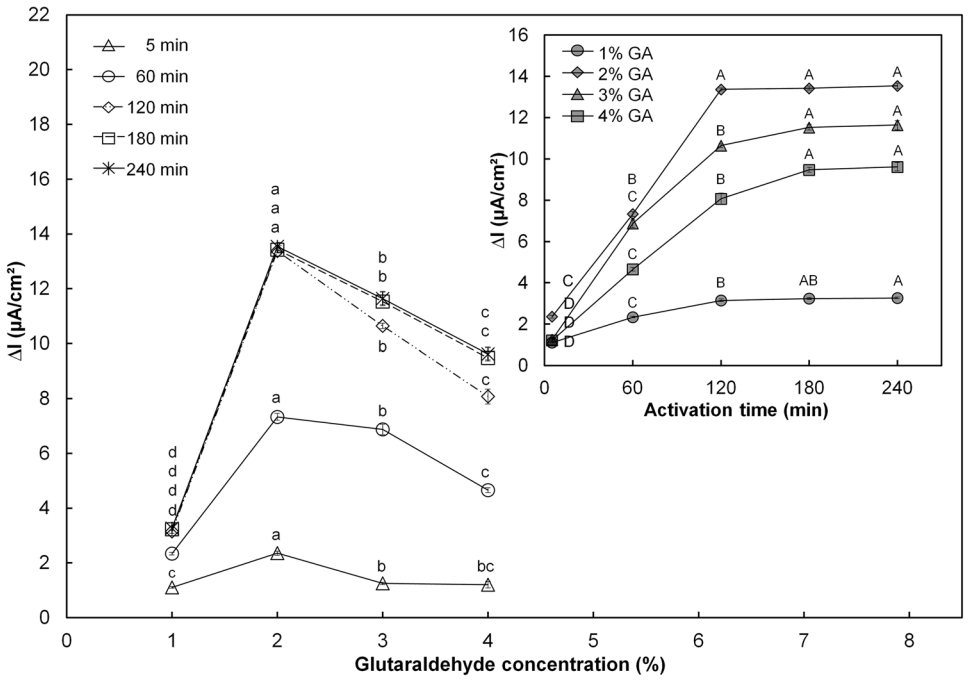

Fig. 2 Effects of glutaraldehyde concentration on responses of developed bienzymatic FIA biosensors to 0.3 mM aspartame. Inset shows effect of activation time on sensor response. Different lowercase letters indicate significant ($p < 0.05$) differences between GA concentrations at an activation time. Different uppercase letters indicate significant ($p < 0.05$) differences between activation times at a GA concentration

Effects of amounts of enzymes and immobilization time on sensor response

The effects were investigated of the amounts of CHY and AOX on the responses of the developed aspartame biosensors. As shown in Fig. 3A, the sensor response significantly increased with an increasing amount of CHY from 50 to 250 U/mL and then slightly increased with the increase in the amount of CHY to 500 U/mL. Therefore, 250 U/mL was chosen as the most suitable amount of CHY for fabrication of the aspartame biosensor. When 50 U/mL of CHY was used, the increase in the amount of AOX from 50 to 100 U/mL did not significantly increase the sensor response to 0.3 mM aspartame. However, when using 250 and 500 U/mL of CHY with an increased amount of AOX from 50 to 100 U/mL, the sensor responses to 0.3 and 0.5 mM aspartame significantly increased. Therefore, 250 U/mL of CHY and 100 U/mL of AOX were determined as the optimal amounts of enzymes for fabrication of the aspartame biosensor in the present study.

The biosensor response is controlled by either the enzyme kinetics or mass transport (Mulchandani et al., 1999). When amounts of enzyme below the saturation value are immobilized, the sensor response is strongly influenced by the amount of immobilized enzyme. However, with a saturation amount of immobilized enzyme, the sensor response is no longer dependent on the immobilized enzyme amount (Mulchandani et al., 1999). Additionally, overloading the enzyme may lead to a decrease in sensor response due to the steric hindrance of enzyme molecules on the support surface and the occurrence of undesirable protein–protein interactions (Ibrahim et al., 2013).

The effect of enzyme immobilization time on sensor response was also studied. The enzyme immobilization was conducted using 250 U/mL CHY and 100 U/mL AOX with varying immobilization times. With the increase in enzyme immobilization time from 5 to 180 min, the sensor response significantly increased and reached a maximum value at 180 min (Fig. 3B). Thereafter, as the enzyme immobilization time was increased above 180 min, there was a slight but significant decrease in the sensor response. The longer coupling reaction time may have resulted in alteration of the degree of unfolding of the enzyme molecule, leading to an enzyme conformational change which may have caused enzyme inactivation (Pan et al., 2015). A similar tendency was reported by Singh et al. (2017). In the present study, 180 min was sufficient time for covalent immobilization of CHY and AOX on the activated supports during fabrication of the bienzymatic FIA biosensor for aspartame.

Optimization of operational conditions for biosensor

The bienzymatic FIA biosensors for aspartame were fabricated using the optimal preparation conditions

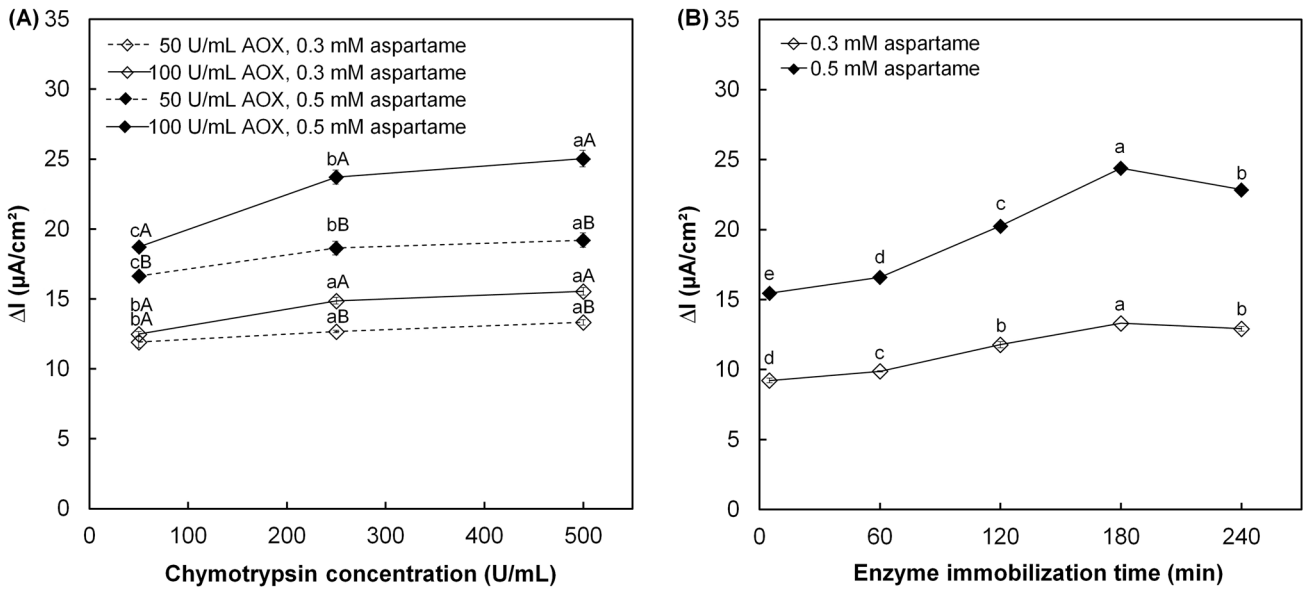

Fig. 3 (**A**) Effects of amounts of enzymes on responses of developed bienzymatic FIA biosensors to 0.3 and 0.5 mM aspartame at enzyme immobilization time of 180 min. Different lowercase letters indicate significant ($p < 0.05$) differences between amounts of chymotrypsin (CHY) at an amount of alcohol oxidase (AOX). Different uppercase letters indicate significant ($p < 0.05$) differences between amounts of AOX at an amount of CHY. (**B**) Effect of enzyme immobilization time on responses to 0.3 and 0.5 mM aspartame observed with developed bienzymatic FIA biosensors fabricated with 250 U/mL CHY and 100 U/mL AOX. Different letters indicate significant ($p < 0.05$) differences between enzyme immobilization times

determined above (2% GA with 120 min activation time; 250 U/mL CHY and 100 U/mL AOX with 180 min immobilization time) and the effects of operational parameters (flow rate, pH, and temperature) on sensor response were investigated.

Effect of flow rate on sensor response

Since the flow rate is an important parameter for operating the biosensor in a flow injection analysis system, the effect of flow rate on the response of the developed aspartame biosensor was investigated. Figure 4A shows the sensor responses toward 0.3 and 0.5 mM aspartame and the sample throughput obtained at different flow rates. As the flow rate increased from 0.2 to 0.6 mL/min, the responses of the developed aspartame biosensor to 0.3 and 0.5 mM aspartame significantly decreased. A similar tendency was found by Polan et al. (2010). Although a higher sensor response was observed at a lower flow rate (0.2 or 0.3 mL/min), fewer samples (less than 5 samples) could be analyzed per hour. Operating the developed aspartame biosensor at a higher flow rate resulted in a higher sample throughput, even though for flow rates above 0.5 mL/min there was a risk of leakage from the high pressure occurring as the carrier buffer passed through the small enzyme reactor. When the flow rate was increased from 0.4 to 0.5 mL/min, the

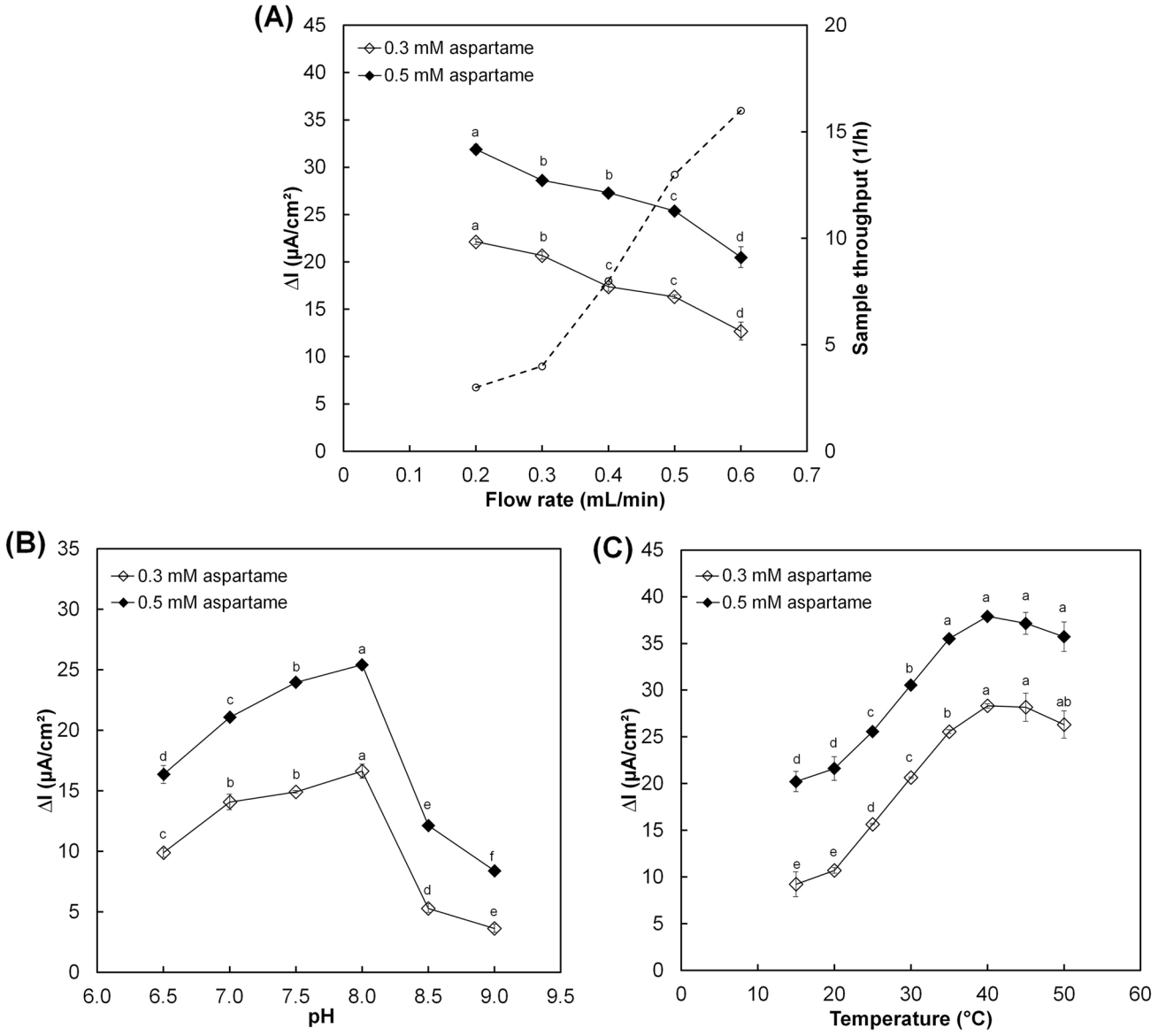

Fig. 4 Effects of (**A**) flow rate, (**B**) pH, and (**C**) temperature on responses of developed bienzymatic FIA biosensors to 0.3 and 0.5 mM aspartame. Different letters indicate significant ($p < 0.05$) differences between (**A**) flow rate, (**B**) pH, and (**C**) temperature

sensor response slightly decreased but the sample throughput increased almost twofold. Therefore, 0.5 mL/min was selected as the optimal flow rate in the present study for operating the developed aspartame biosensor with a sample throughput of 12–13 samples/h because it provided a good compromise among the factors of the magnitude of sensor response, the speed of measurement and buffer consumption.

The sensor response relies on the combination effect of the enzymatic reaction rate, the mass transfer rate to the electrode surface, and the electrochemical reduction or oxidation of the compound involved in the enzymatic reaction (Ortega et al., 1994). Since increasing the flow rate results in an increase in mass transfer, the reduction in sensor response with increasing flow rate may be caused mainly by the limitation of the enzymatic reaction (Ortega et al., 1994). In flow injection analysis, dispersion or dilution takes place when the injected sample is carried toward the detector by a carrier stream and both the shape and height of the response peak are influenced by dispersion (Ghous, 1999). When a higher flow rate is used, a narrower peak is obtained and more samples can be determined, while the residence time of the analyte within the enzyme reactor is shorter, resulting in a reduced sensor response (Kulkarni and Vaidya, 2015). At a lower flow rate, a wider peak is obtained and a limited number of samples can be analyzed, while the residence time is longer, resulting in a more complete enzymatic reaction, which ultimately increases the sensor response (Adányi et al., 2004).

Effect of pH on sensor response

Similar to other proteins, enzymes have a tertiary structure that is sensitive to pH change and can function best within an optimal pH range, while they are denatured at extreme pH values (Rye et al., 2016). Thus, the effect of pH was investigated on the responses of the developed bienzymatic FIA biosensor to 0.3 mM and 0.5 mM aspartame and the results are depicted in Fig. 4B. The sensor responses significantly increased with increasing pH from 6.5 to 8.0. The maximum sensor response was achieved at pH 8.0; for greater pH values there was a significant decrease in the sensor response. The overall enzymatic activity is influenced by the pH of the surrounding environment or solutions containing substrates (Rye et al., 2016). Amino acid residues at the enzyme active site are sensitive to pH change that can disrupt the way substrate molecules bind and have their own characteristics (acidic or basic) that are suitable for catalysis (Rye et al., 2016). The optimal pH for soluble CHY has been reported to be close to 7.8 (Ásgeirsson and Bjarnason, 1991), while the optimal pH values for soluble AOX have been reported to be 7.5 (Couderc and Baratti, 1980) and 8.0 (Yildiz and Toppare, 2006). Chou (1996) reported that the optimal pH was in the range 6.5–8.5 for an aspartame biosensor prepared using CHY and AOX.

In the present study, 8.0 was selected as the optimal pH for operating the developed bienzymatic FIA biosensor for aspartame based on immobilized CHY and AOX. This optimal pH was close to those of free CHY and AOX, suggesting that immobilization may not alter the pH of the enzyme microenvironment. It is known that immobilization could affect pH shifts in enzymatic activity and the optimal pH may shift either way (Lartigue, 1975). Yildiz and Toppare (2006) reported that the optimal pH for enzyme immobilized in a negatively charged matrix shifted to alkaline due to the accumulation of protons near the negatively charged matrix, resulting in a decreased pH in the enzyme microenvironment compared to the pH of the bulk solution.

Effect of temperature on sensor response

The effect of temperature on the responses of the developed bienzymatic FIA biosensor to 0.3 and 0.5 mM aspartame is shown in Fig. 4C. The sensor response significantly increased with increasing temperature from 15 to 40 °C and reached a maximum value at 40 °C and thereafter slightly decreased. Chou (1996) developed an aspartame biosensor based on co-immobilized CHY and AOX on a dissolved oxygen electrode and reported a decrease in dissolved oxygen or an increase in sensor response with increasing temperature from 25 to 60 °C. The optimum temperature for free CHY is 50 °C (Fernández et al., 2002) and for free.

AOX is 37 °C (Couderc and Baratti, 1980). In general, the initial rate of enzymatic reaction is dependent on temperature according to the Arrhenius equation:

$$\text{rate} = A\mathrm{e}^{-E/RT} \tag{3}$$

where, A is a constant known as the pre-exponential factor, E is the activation energy (J/mol), R is the gas constant (8.2 J/mol K), and T is the absolute temperature (K) (Wilson, 2010). When the temperature was increased, the initial increase in sensor response was observed due to increases in the enzymatic reaction and mass transfer rates; however, if the temperature is too high, denaturation of enzymes may occur and they may lose their activity (Mulchandani et al., 1999; Wilson, 2010), resulting in a decrease in sensor response.

Despite the highest sensor response was observed at 40 °C, room temperature (25–30 °C) was selected as the optimal temperature for operating the aspartame biosensor developed in the present study. Although operation of the developed aspartame biosensor at room temperature only resulted in a moderately high response, this could also prolong the stability of biosensor. Operating the biosensor at high temperature may deteriorate the stability of the

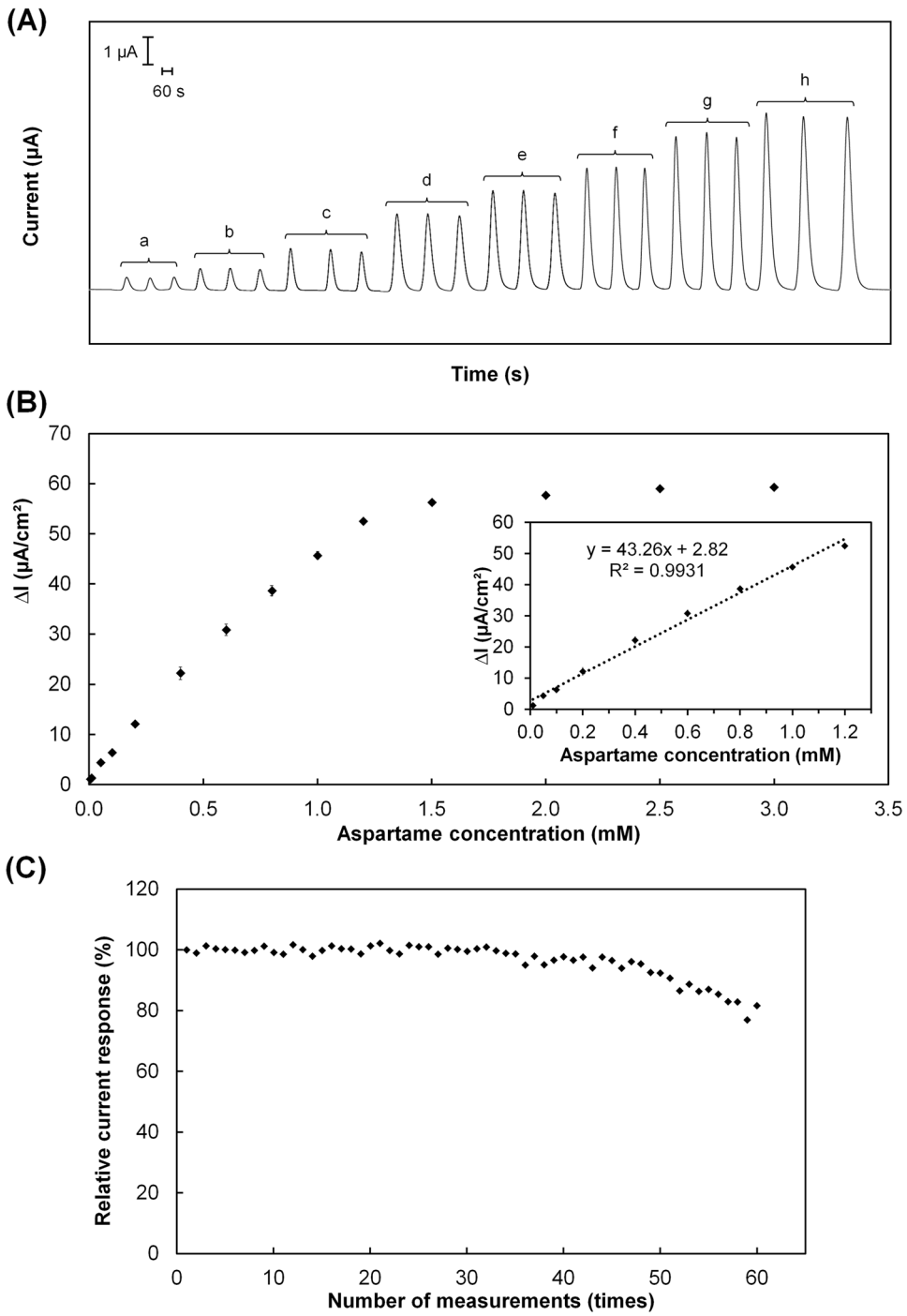

Fig. 5 (**A**) Amperometric current signals for successive triplicate injections of (a) 0.05 mM, (b) 0.1 mM, (c) 0.2 mM, (d) 0.4 mM, (e) 0.6 mM, (f) 0.8 mM, (g) 1.0 mM, and (h) 1.2 mM aspartame into developed bienzymatic FIA biosensor system for aspartame. (**B**) Current responses of developed bienzymatic FIA biosensor toward aspartame in the concentration range 0.005–3.0 mM. Inset in (**B**) shows calibration curve for aspartame. (**C**) Operational stability of developed bienzymatic FIA biosensor

biosensor. Wong et al. (2017) studied the thermostability of immobilized chymotrypsin and observed that increasing the incubation temperature in the range 30–60 °C led to a markedly decrease in thermostability. In addition, Göktuğ et al. (2005) studied the thermal stability of a lactose biosensor and found that increasing the incubation temperature in the range investigated (30–40 °C) resulted in a noticeable reduction in thermal stability.

Analytical performance of the developed bienzymatic FIA biosensor for aspartame

The amperometric current signals (in triplicate) obtained from the developed bienzymatic FIA biosensor for aspartame are shown in Fig. 5A. Figure 5B demonstrates the resulting current responses of the developed aspartame biosensor in the concentration range 0.005–3.0 mM. The

Table 1 Aspartame content in beverage samples determined using developed aspartame biosensor compared to HPLC method

Sample	Aspartame concentration (mg/kg) [a]		Relative error (%) [b]	*p* value
	Biosensor	HPLC		
Soft drink	156.95 ± 3.13	154.85 ± 2.55	1.36	ns
Orange juice with nata de coco	115.02 ± 4.55	117.90 ± 1.59	− 2.44	ns
Peach and white grape juice with yoghurt and nata de coco	126.64 ± 2.82	133.45 ± 0.88	− 5.10	ns

ns not significant

[a]Mean ± SD (n = 3)

[b]Relative error (%) = 100 × (biosensor value − HPLC value)/HPLC value

calibration curve for aspartame obtained from the developed biosensor is depicted in the inset of Fig. 5B. The proposed bienzymatic FIA biosensor for aspartame showed a linear range from 0.01 to 1.2 mM with a coefficient of determination (R^2) of 0.9931, a sensitivity of 43.26 μA/mM·cm^2 and a detection limit of 0.005 mM (defined as a signal-to-noise ratio of 3). The relative standard deviations (%RSD) of the sensor responses to 1.0 and 1.2 mM aspartame were 1.60 and 1.17%, respectively, and the response and recovery times were 126–131 s and 220–240 s, respectively. The bienzymatic FIA biosensor for aspartame developed in the present study demonstrated good sensitivity and had analytical performance with regard to the linear range and detection limit comparable to other reported aspartame biosensors (Kirgöz et al., 2006; Male et al., 1991; Male et al., 1993; Villarta et al., 1993). The detection limit of other previously reported biosensors for aspartame is in the range 0.0002–0.15 mM (Compagnone et al., 1997; Male et al., 1991; Male et al., 1993; Radulescu et al., 2014; Villarta et al., 1993). While other methods for determination of aspartame such as ion chromatography (Chen and Wang, 2001), HPLC (Trandafir et al., 2009), spectrophotometry (Fatibello-Filho et al., 1999), liquid chromatography (Üstün Özgür and Kasapoğlu, 2019), and capillary electrophoresis (Bergamo et al., 2011) have a reported detection limit in the range 0.03–4.2 μg/mL (0.0001–0.014 mM).

Operational stability of biosensor

The operational stability of the biosensor was investigated based on 60 successive measurements of 0.1 mM aspartame with the developed bienzymatic FIA biosensor for aspartame over a 10 h period. The developed biosensor could be repeatedly used for at least 44 measurements under the optimal operating conditions with a very small loss of 2% compared to the original response. After 60 consecutive assays, the sensor retained 82% of its initial current response (Fig. 5C). The developed aspartame biosensor showed good stability for repeated measurements of aspartame.

Determination of aspartame in real food samples

The developed bienzymatic FIA biosensor was utilized to determine aspartame concentrations in beverage samples (soft drink and 25% fruit juices). The results obtained from the developed aspartame biosensor were compared to those obtained using the HPLC method (Table 1). The aspartame concentrations detected by the developed FIA biosensors were not significantly different from those determined using the HPLC method. Thus, it was concluded that the bienzymatic FIA biosensor developed in the present study could be satisfactorily applied for aspartame determination in real food samples. Furthermore, the developed bienzymatic FIA biosensor could be used as an alternative tool for rapid and accurate analysis of aspartame in food and beverage products.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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