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ORIGINAL PAPER

Use of some amino acid potentiometric biosensors as detectors in ion chromatography

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Abstract

In this study, composite amino acid–sensitive potentiometric biosensors in microsizes were prepared and used as detectors in ion chromatography (IC). First, ammonium–selective electrodes were prepared, and their potentiometric behavior was tested. Then, the L–amino acid oxidase (L–AAO) enzyme was attached to the surface of the ammonium–selective potentiometric electrodes with glutaraldehyde crosslinker. The potentiometric behavior of the prepared biosensors against different L–amino acids was investigated. The biosensors exhibited ideal potentiometric responses to some of the L–amino acids. Biosensors with the ideal potentiometric behavior were placed in the moving medium flow cell together with the Ag/AgCl reference electrode. This flow cell was placed at the exit of the column in the ion chromatographic system, allowing it to function as a detector. The chromatograms were obtained by injecting free amino acids into the prepared chromatography–potentiometry hybrid system. The obtained results showed that the prepared biosensors can be used as detectors in chromatography and as detectors in the determination of free amino acids.

Keywords Amino acid · Potentiometry · Ion chromatography · Biosensor

Introduction

Amino acids (AAs) are compounds that contain amino ($-NH_2$) and carboxyl groups ($-COOH$) in their structures, and have many biological activities such as body growth, tissue regeneration, gene expression, preventing tumorigenesis, suppressing obesity and reducing blood pressure (Kowalska et al. 2022; Tůma 2021). Naturally occurring L–amino acids are required for protein synthesis, and most of the proteinogenic amino acids found in humans are found in human plasma at concentrations of μM (Peace and Gilani 2005; Ferré et al. 2019). Half of the 20 amino acids that enter the protein structure cannot be synthesized by humans or adequately synthesized. Non-synthesized amino acids must be obtained from the diet (Sharer et al. 2018). To date, the determination of amino acids in food, chemical and biological samples has been carried out using analytical methods such as liquid chromatography–tandem mass spectrometry (LC–MS/MS) (Song et al. 2015; Kambhampati et al. 2019), gas chromatography–mass spectrometry (Jiménez-Martín et al. 2012), high–performance liquid chromatography (HPLC) (Zhao et al. 2013) and capillary electrophoresis (CE) (Manaenkov et al. 2003; Tůma et al. 2022; Tůma 2022). Xu et al. (2020) examined these analytical methods used in amino acid determination among each other and compared with HPLC, LC–MS and GC–MS are more sensitive and more effective; however, they reported that HPLC is more cost-effective.

Potentiometry–based sensors or biosensors have significant advantages such as wide linear range, low detection limit, ease of use, low cost, high selectivity and sensitivity, long lifetime and fast response time (Özbek and Isildak 2022a, b, c). Because of these advantages, potentiometry is an important topic emphasized by researchers. Biosensors can be defined as analytical devices developed via combination of the selectivity properties of biological molecules with the processing capability of modern electronic techniques by utilizing the knowledge of many disciplines

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such as biology, chemistry, biochemistry and engineering (Shruthi et al. 2014). Biosensors have been applied in many fields, namely, food quality, biotechnology, environmental monitoring, health care, personal safety and security, and they provide better stability and sensitivity as compared with the traditional analytical methods (Metkar and Girigoswami 2019; Mehrotra 2016; Özbek et al. 2022a). In this study, microsized potentiometric amino acid biosensors were prepared and developed into microflow cells and included in the ion chromatography system. Thus, they were used as detectors for the determination of free amino acids.

Experimental

Chemicals and reagents

1,4–Diaminobutane, triethylamine, methanol used in PVC–NH_2 synthesis were obtained from Merck. Amino acid standards [Alanine (Ala), Arginine (Arg), Aspartic acid (Asp), Phenylalanine (Phe), Glycine (Gly), Glutamic acid (Glu), Glutamine (Gln), Histidine (His), Isoleucine (Ile), Lysine (Lys), Leucine (Leu), Tyrosine (Tyr), Valine (Val)] are obtained from Fluka, and all are in chromatographic purity. High molecular weight PVC, bis(2-ethylhexyl) sebacate (BEHS), potassium tetrakis(*p*-chlorophenyl)borate (KT*p*ClPB), L–amino acid oxidase (L–AAO), glutaraldehyde, tetrahydrofuran (THF), dicyclohexano-18-crown-6 and metal nitrate salts were purchased from Merck and Sigma Aldrich. Graphite, epoxy (Macroplast Su 2227) and hardener (Desmodur RFE), used to prepare conductive solid state, were obtained from Sigma Aldrich, Henkel (Istanbul, Turkey) and Bayer AG (Darmstadt, Germany), respectively.

Instruments

Potentiometric measurements were taken using on a computer–controlled multichannel potentiometric system (ISEDO Medical, Turkey). Ag/AgCl reference electrode (Thermo Orion) was used in this measurement system. pH measurements were taken with a digital pH meter (Mettler Toledo Model S220–K). Finally, Thermo Fisher Dionex ICS–1100 ion chromatography was used.

Method

In this study, completely all–solid–state contact ammonium–selective potentiometric electrodes were prepared, and amino acids were attached to the surface of these electrodes by using glutaraldehyde as a crosslinker.

Synthesis of PVC–NH_2

PVC–NH_2 was synthesized as given in the literature (Walcerz et al. 1995). Accordingly, 1.43 g of PVC, 6.0 g of 1,6-diamino hexane and 3.5 mL of triethylamine were taken into a balloon. Thirty-five milliliters of methanol was added to this mixture and refluxed for 3.5 h. Then, the yellowish solid formed was washed with methanol, concentrated HCl and water, then washed with methanol again and dried. The dried substance was dissolved in THF. The insoluble part was filtered off, and the soluble part was dried. The dried yellowish polymer material was used as PVC–NH_2.

Preparation of all–solid–state ammonium–selective electrodes

Firstly, a mixture of all–solid–state contact consisting of 50.0% (*w*/*w*) graphite, 35.0% (*w*/*w*) epoxy and 15.0% (*w*/*w*) hardener was thoroughly dissolved in approximately 3 mL of THF. After obtaining the appropriate viscosity, the ends of copper wires were dipped into this mixture several times and covered with conductive solid contact. The coated copper wires were kept in the dark at room temperature for about 24 h (Özbek 2023; Özbek et al., 2022b). Then, ammonium–selective electrodes were prepared. For this purpose, nonactin, dibenzo-18-crown-6, KT*p*ClPB, BEHS and synthesized PVC–NH_2 were dissolved in approximately 3 mL of THF. After reaching a suitable viscosity, the previously prepared solid contact electrodes were dipped into this mixture several times and left to dry.

Preparation of amino acid biosensors

The crosslinker was prepared from 100 µL of 2.5% (*w*/*w*) glutaraldehyde using 400 µL of phosphate buffer ($H_2PO_4^-$/HPO_4^{2-}) at pH 7.02. Enzyme solution was prepared by dissolving 2.0 mg of L–amino acid oxidase enzyme in 500 µL phosphate buffer at 5 mM and pH 7.02. The cocktail prepared by mixing the glutaraldehyde solution, and the enzyme solution was kept in the dark and in the refrigerator (4 °C). The pre–prepared ammonium–selective electrodes were dipped several times in the glutaraldehyde–enzyme cocktail, and the surfaces of the electrodes were coated. Finally, the sensor surfaces were washed with phosphate buffer to remove the residues of the exposed glutaraldehyde molecule that did not adhere to the surface of the biosensors. The steps of attachment of enzymes to the biosensor surface are summarized in Fig. 1.

Preparation of flow cells

Miniaturized flow cells (with microliter dead volume) used as detectors in the ion chromatography system were formed

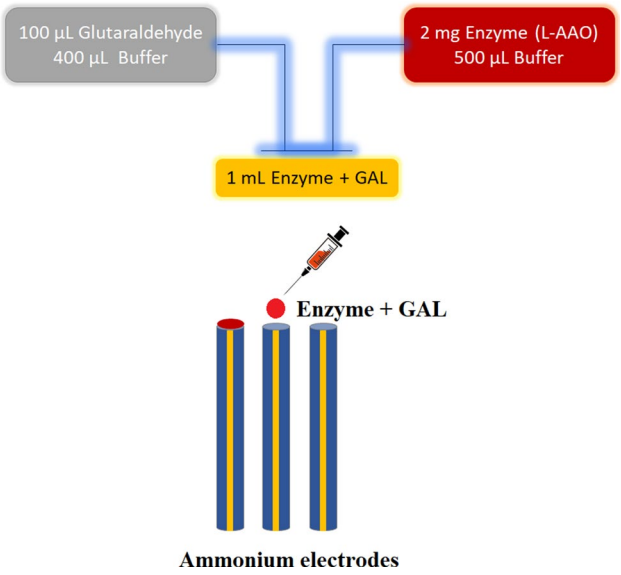

Fig. 1 The steps of attachment of enzymes to the biosensor surface

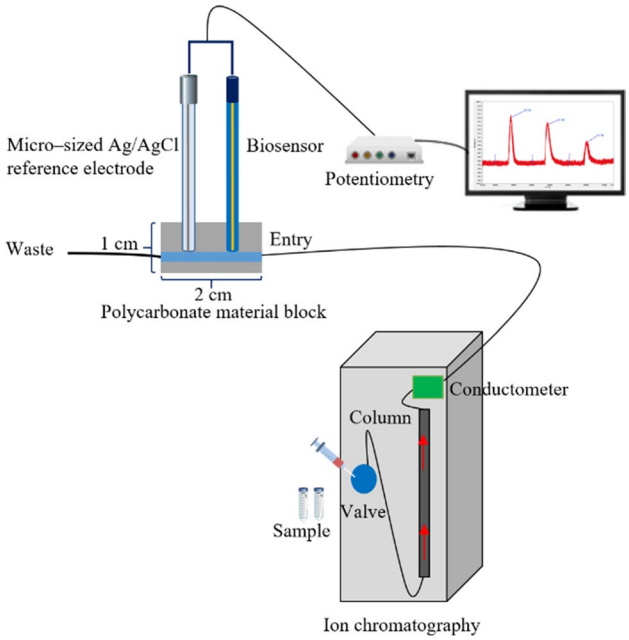

Fig. 2 The prepared ion chromatography–potentiometry hybrid system

from a 2-cm-long, 1-cm-wide and 1-cm-high polycarbonate material block. Microsized Ag/AgCl reference electrode with amino acid–sensitive biosensor was placed on the polycarbonate block. The microsized Ag/AgCI reference electrode included in this system was produced in our laboratory. Ag/AgCl was added directly to the flow cell by adding a tube containing 3.0% Agar + saturated KCl to the tip of the reference electrode. The chromatographic separations were performed without using a suppressor of the ion chromatography device. The free amino acids were determined by connecting the prepared flow cells with a dead volume of microliter to the exit of the separation column like a detector in the ion chromatographic system. Figure 2 shows the ion chromatography–potentiometry hybrid system prepared.

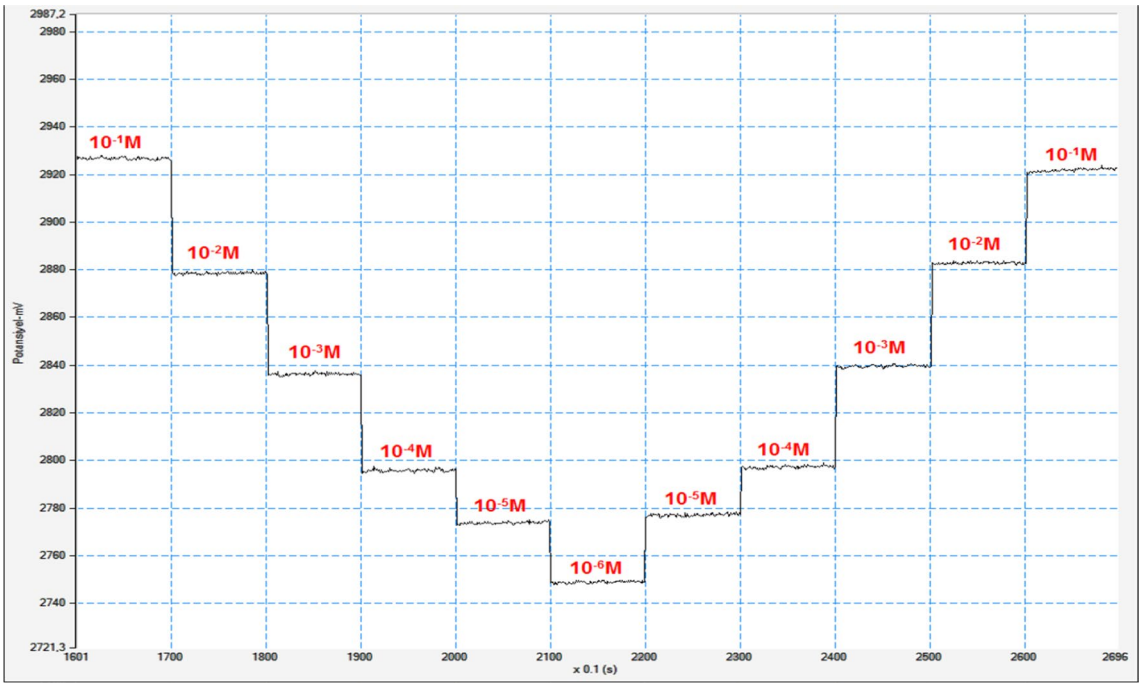

Fig. 3 Potentiometric response of the prepared ammonium–selective electrode

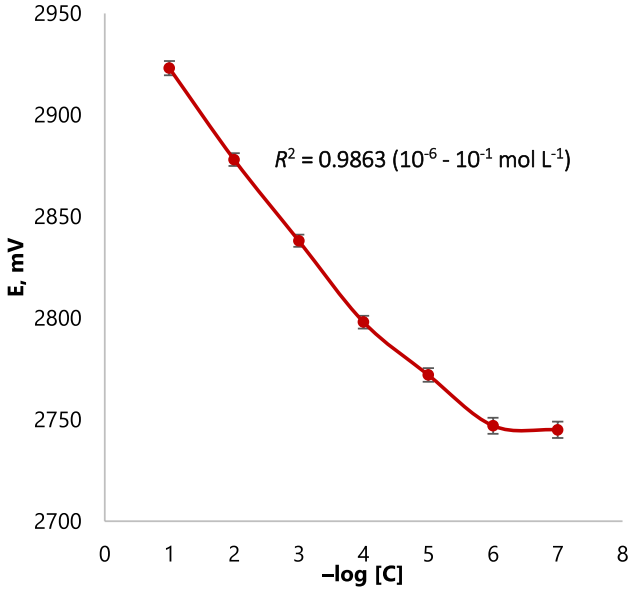

Fig. 4 Calibration curve of ammonium–selective electrode

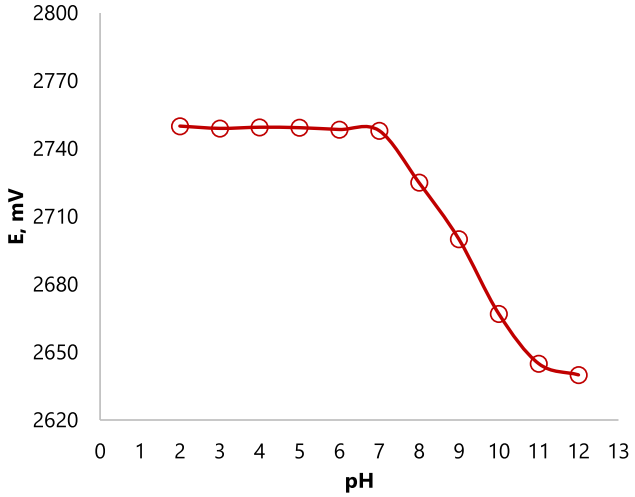

Fig. 5 pH response of the ammonium–selective electrode

Results and discussion

Characterization of ammonium-selective potentiometric electrode

In the preparation of the amino acid biosensor, an all–solid–state ammonium–selective electrode was used as the basic sensor (ion–selective electrode). The ammonium–selective electrode has a composition of PVC–NH_2, nonactin, dibenzo-18-crown-6, anion excluder (KT*p*ClPB) and plasticizer (BEHS) in the ratio of 36.0:2.5:1.0:0.5:60.0 (mg). The potentiometric response of the prepared basic ammonium–selective sensor is given in Fig. 3. The prepared electrode exhibited a wide concentration range from 1.0×10^{-6} to 1.0×10^{-1} mol L^{-1} (R^2: 0.9863). The detection limit of the prepared electrode was calculated using the calibration curve in Fig. 4 by writing the potential value corresponding to the intersection point of the extrapolations on the horizontal and vertical axes in the linear equation. The detection limit of the ammonium–selective electrode was calculated as 8.7×10^{-7} mol L^{-1}. To determine the pH operating range of the ammonium–selective electrode, it was determined by measurements taken in solutions of 1.0×10^{-2} mol L^{-1} constant ammonium

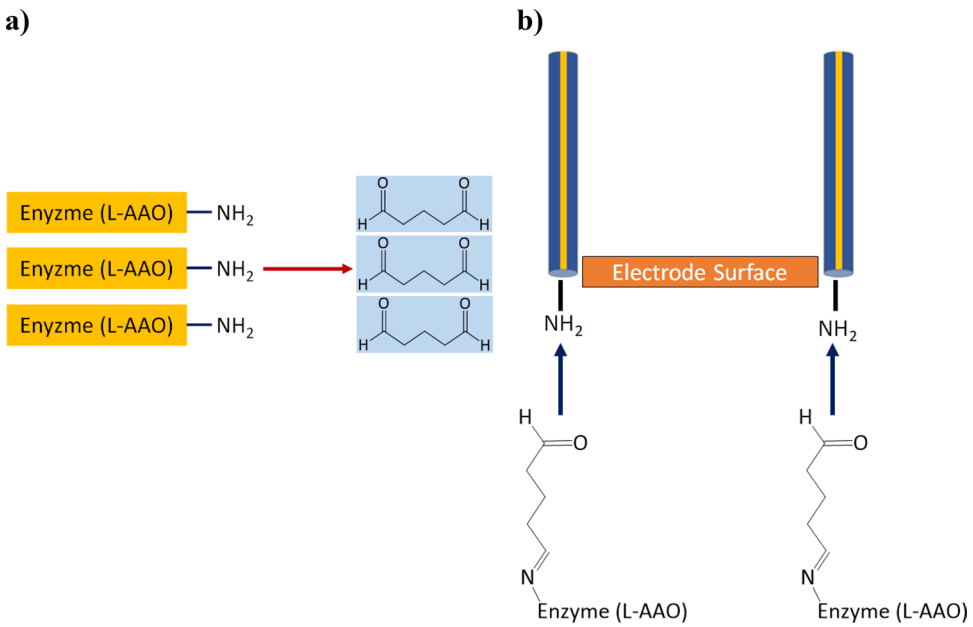

Fig. 6 **a** Binding of glutaraldehyde with enzyme. **b** Attachment of glutaraldehyde–bound enzyme molecule (L–AAO) to the surface of the ammonium electrode

Table 1 Potentiometric performance characteristics of the biosensor against amino acid solutions prepared with deionized water

Amino acid	Linear concentration range (mol L^{-1})	Limit of detection (mol L^{-1})	Slope (mV/decade)	R^2
Ala	1.0×10^{-1} – 1.0×10^{-4}	7.74×10^{-5}	42.2	0.9962
Arg	Not response			
Asp	Not response			
Phe	1.0×10^{-1} – 1.0×10^{-3}	7.47×10^{-4}	34.0	0.9928
Gly	Not response			
Glu	1.0×10^{-1} – 1.0×10^{-4}	1.80×10^{-5}	18.9	0.9831
Gln	1.0×10^{-2} – 1.0×10^{-5}	9.75×10^{-6}	48.2	0.9806
His	1.0×10^{-1} – 1.0×10^{-3}	5.03×10^{-4}	39.1	0.9910
Ile	1.0×10^{-2} – 1.0×10^{-4}	9.80×10^{-5}	38.5	0.9990
Lys	1.0×10^{-1} – 1.0×10^{-5}	5.27×10^{-6}	26.8	0.9944
Leu	Not response			
Tyr	1.0×10^{-1} – 1.0×10^{-3}	8.39×10^{-4}	10.5	0.9990
Val	1.0×10^{-1} – 1.0×10^{-3}	4.17×10^{-4}	29.4	0.9990

Table 2 Potentiometric performance characteristics of the biosensor against amino acid solutions prepared with phosphate buffer (pH: 7.02)

Amino acid	Linear concentration range (mol L^{-1})	Limit of detection (mol L^{-1})	Slope (mV/decade)	R^2
Ala	1.0×10^{-1} – 1.0×10^{-3}	5.16×10^{-5}	10.5	0.9948
Arg	1.0×10^{-3} – 1.0×10^{-5}	8.67×10^{-4}	36.5	0.9990
Asp	Not response			
Phe	1.0×10^{-1} – 1.0×10^{-3}	7.74×10^{-4}	27.0	0.9838
Gly	1.0×10^{-1} – 1.0×10^{-3}	7.43×10^{-4}	41.0	0.9806
Glu	1.0×10^{-1} – 1.0×10^{-3}	9.60×10^{-4}	17.5	0.9954
Gln	1.0×10^{-1} – 1.0×10^{-3}	5.74×10^{-4}	51.0	0.9990
His	1.0×10^{-1} – 1.0×10^{-3}	6.17×10^{-4}	52.0	0.9990
Ile	1.0×10^{-1} – 1.0×10^{-3}	4.70×10^{-4}	51.0	0.9980
Lys	1.0×10^{-2} – 1.0×10^{-5}	8.02×10^{-5}	45.8	0.9937
Leu	1.0×10^{-1} – 1.0×10^{-4}	8.31×10^{-5}	13.0	0.9803
Tyr	Not response			
Val	1.0×10^{-1} – 1.0×10^{-3}	4.50×10^{-4}	45.5	0.9980

concentration and 5.0×10^{-3} mol L^{-1} phosphate buffer with pH ranging from 2.0 to 12.0. The pH dependence of the ammonium–selective electrode is shown in Fig. 5. The decrease in potential values after pH > 7.0 can be attributed to the formation of ammonia due to the pH change of the solution. The potentiometric selectivity of ammonium–selective electrode was studied in the presence of K^+, Na^+ and Ca^{2+} ions. The potentiometric selectivity coefficients were calculated using the separate solution method (SSM) (Umezawa et al. 2000). The selectivity

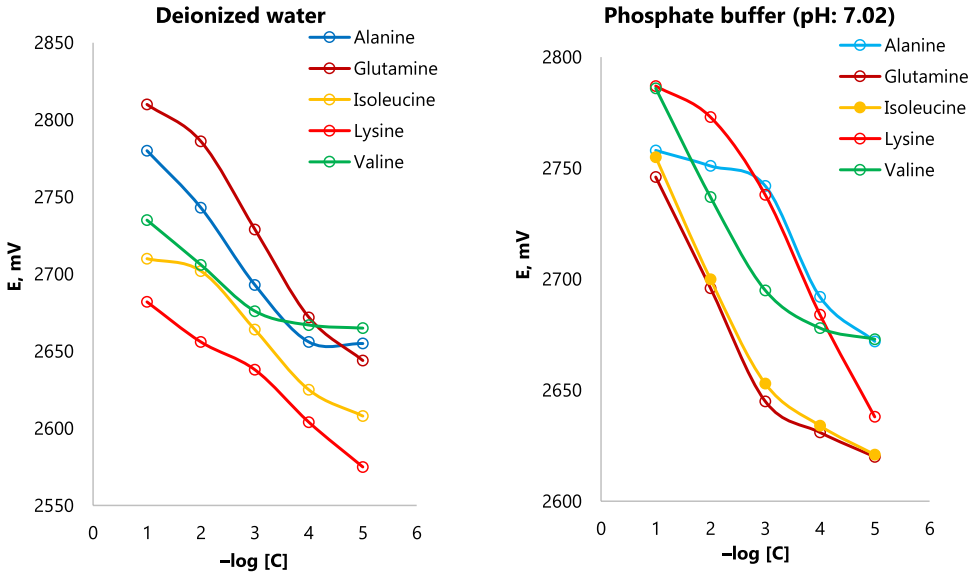

Fig. 7 The calibration curve of the biosensor against amino acid solutions

coefficients were calculated as –1.85, –3.18 and –3.01 for K^+, Na^+ and Ca^{2+}, respectively. The response time of the electrode was determined according to IUPAC recommendations (Buck and Lindner 1994). The response time of the prepared electrode was investigated at various concentrations from 1.0×10^{-1} to 1.0×10^{-5} mol L^{-1} of the ammonium solutions. The prepared electrode equilibration time was determined as 5 s for each tenfold concentration value. According to these data, it is clear that the ammonium–selective electrode can be successfully applied as a basic sensor in the production of biosensors.

Potentiometric performance characteristics of amino acid biosensors

After testing the potentiometric performance of the ammonium–selective electrodes, the enzymes (L–AAO) were coupled with the crosslinking reagent glutaraldehyde. The binding of enzymes to ammonium–selective electrodes is summarized in Fig. 6.

The potentiometric behavior of the prepared biosensor against amino acid solutions was examined using deionized water and phosphate buffer (pH: 7.02). Potentiometric measurements were taken using solutions of amino acids in the concentration range of 1.0×10^{-1} – 1.0×10^{-5} mol L^{-1}. The potentiometric linear working range, detection

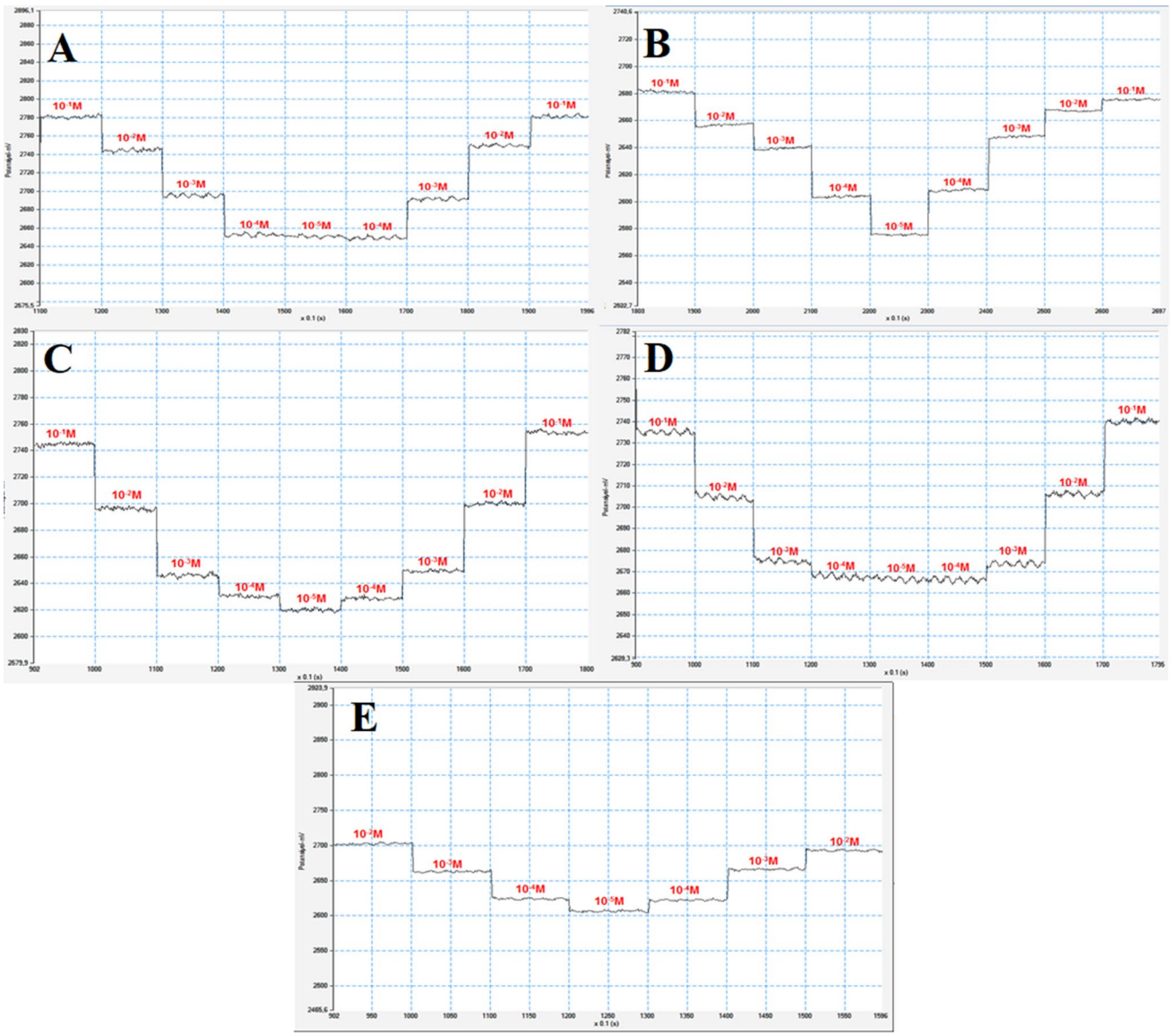

Fig. 8 The potentiometric response of biosensor against **a** Ala, **b** Lys, **c** Glu, **d** Val, **e** Ile (deionized water)

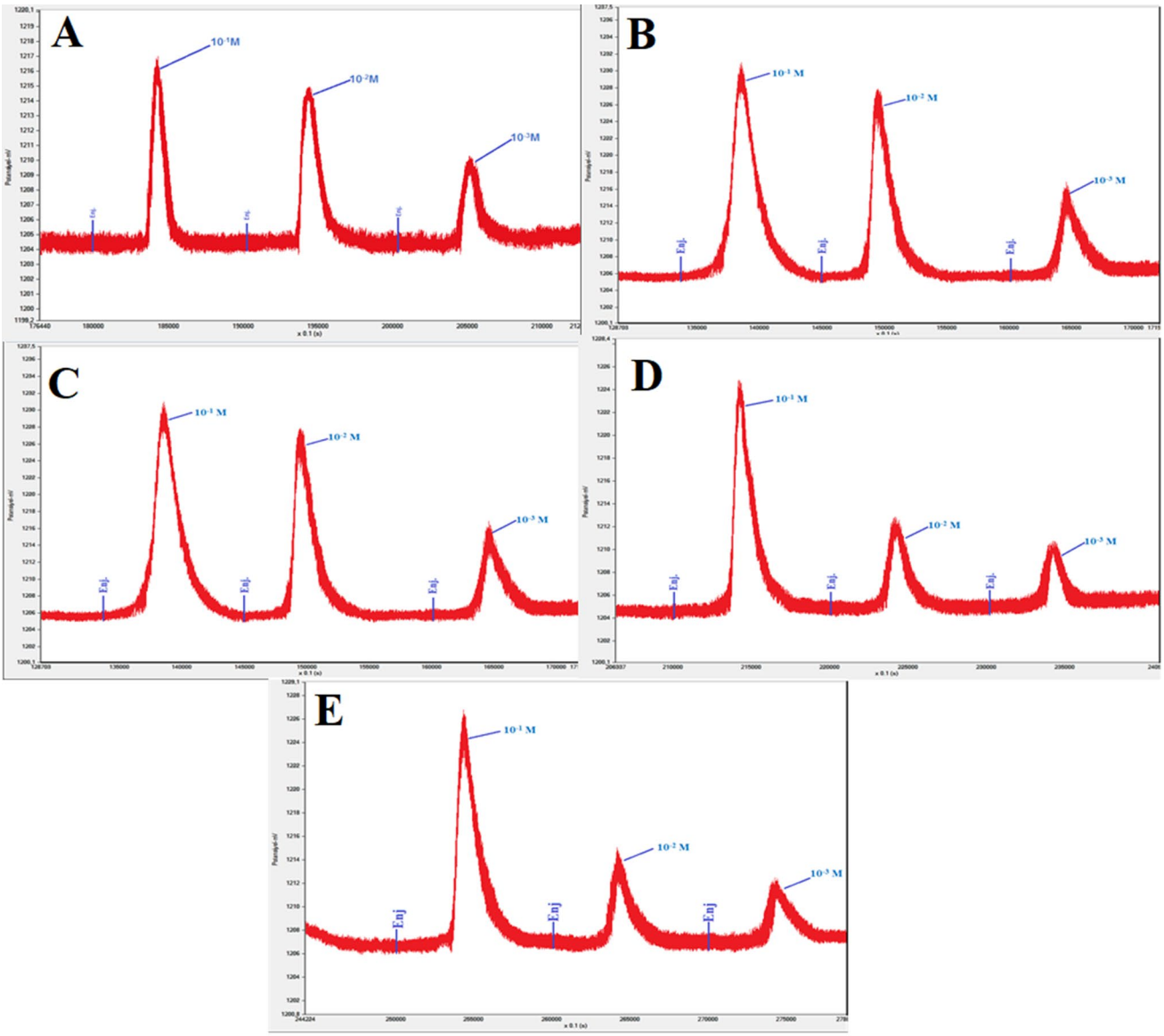

Fig. 9 The chromatograms obtained as a result of injection of 1.0×10^{-1} – 1.0×10^{-3} mol L^{-1} **a** Ala, **b** Lys, **c** Glu, **d** Val, **e** Ile solutions

limit, slope and R^2 values of the biosensor against amino acid solutions are given in Table 1 and Table 2. The calibration curve of amino acids with the widest working range is given in Fig. 7. The potential response of the biosensor to the amino acids to which it exhibits a linear response is given in Fig. 8. When Figs. 7, 8 and Tables 1, 2 are examined, it is clearly seen that the biosensor exhibits a more ideal potentiometric response in a wider working range against amino acid solutions prepared with deionized water. On the other hand, the biosensor exhibited a more noisy potentiometric response in measurements taken against amino acid solutions prepared with phosphate buffer (pH: 7.02). This may be due to the presence of possible interference ions in the phosphate buffer.

Determination of amino acids using potentiometry–ion chromatography hybrid method

The free amino acids were determined by connecting the prepared flow cells with a dead volume of microliter to the exit of the separation column like a detector in the ion chromatographic system. The most suitable flow parameters were determined by testing the mobile phase composition and flow rate for the system. In this study, 6.68×10^{-3} mol L^{-1} sodium phosphate buffer with a pH of 6.9 was used as the mobile phase. The flow rate was determined as 1.00 mL/min, and potentiometric chromatograms were obtained by injecting standard amino acid solutions in the 1.0×10^{-1}–1.0×10^{-3} mol L^{-1} concentration

range into the system (Fig. 9). When the chromatograms obtained using the biosensor as a detector in the ion chromatography–potentiometry hybrid system are examined, it is clearly seen that it shows a linear operating range in the concentration range of 1.0×10^{-1}–1.0×10^{-3} mol L^{-1} for Ala, Lys, Glu, Val, Ile (Fig. 9). In the chromatograms, the peak heights decreased against decreasing concentrations and the peak areas decreased with decreasing concentration. As a result, the prepared biosensors can be used as detectors in the chromatographic analysis of amino acids, and they can also perform amino acid determinations in this concentration range.

Conclusion

In this study, microsized amino acid–sensitive biosensors were prepared and microflow cells were developed and adapted to the ion chromatographic system. For this purpose, microsized composite ammonium-selective sensors to be used as basic sensors in amino acid-sensitive biosensors were prepared, and their potentiometric performance characteristics were determined. The L–amino acid oxidase enzyme was covalently attached to the surface of the ammonium–selective sensors, which was predicted to be suitable for preparing biosensors, using glutaraldehyde crosslinker. The prepared flow cells were mounted at the column outlet in the liquid chromatographic system and were allowed to act as a detector. The most appropriate flow rate and mobile phase type were determined at the end of the experiments. Biosensors were placed in these flow cells, and chromatograms were obtained as a result of injection of amino acids. According to the results obtained from the chromatograms, it was determined that the biosensor could be used as a detector in the ion chromatography system.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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