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# AMPEROMETRIC LYSINE BIOPROBES ANALYSIS IN FEEDS

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**Summary**—Amperometric enzyme electrode probes have been constructed for the specific determination of *L*-lysine and used in batch and flow analysis. The enzyme lysine oxidase was immobilized on a preactivated polymer support which was placed on a platinum electrode. Additional blocking membranes conferred high stability, reproducibility and avoided electrochemical and enzyme interferences. Parameters including pH, temperature, storage and operational times were optimized. Lysine was determined in the range $10^{-6}$–$2.10^{-3}M$ with a detection limit of $5 \times 10^{-7}M$. The Michaelis constant was $2 \times 10^{-3}M$. This value was approximately two order of magnitudes higher than that reported in literature for the free enzyme. The response time of the probe was about 2 min in batch and flow analysis and 30 sec in flow injection analysis (FIA). The resulting probes were stable for more than three months with more than 300 analyses performed. The determination of lysine was carried out by both flow-through analysis and FIA. Analysis in feeds was carried out by acid hydrolysis to liberate lysine; then the solution was analyzed by the bioprobe and HPLC procedures. Results by the two methods correlated well.

The increasing demand in many countries for regulatory rules on the nutritional values for proteins has led scientists to develop new, rapid and specific methods for the determination of essential aminoacids in food and feeds. In this context, *L*-lysine, an essential amino acid is an important indicator of the nutritional quality of food.[1,2]

Several analytical methods for quantitative determination of lysine have been proposed, including reverse-phase liquid chromatography with precolumn derivatization,[3] liquid chromatography with fluorescence detection via precolumn dansylation,[4] aminoacid analysis[5] and paper electrophoresis.[6] These methods often do not satisfy the requirement for fast, accurate and specific analysis. Moreover they require expensive instrumentation and reagents. Electroanalytical methods based on the use of a $CO_2$ electrode and the enzyme lysine decarboxylase,[7] or using a platinum electrode coupled to *L*-lysine dehydrogenase have been reported.[8]

The first electrode, however, suffers from interference by atmospheric $CO_2$ and has a high detection limit ($10^{-4}M$). The second requires the cofactor $NAD^+$ and the mediator ferricyanide ion in solution. This makes the analysis more complicated with the potential problem of collateral reactions when the probe is used in a real matrix.

Recently a flow-through analyzer was described for lysine analysis using a Clark-type oxygen electrode and *L*-lysine-2-monooxygenase immobilized on silica gel.[9]

Romette *et al.*[10] used a Clark $O_2$ electrode coupled with *L*-lysine oxidase Li *et al.* used an optical sensor for determining lysine.[11]

Our system is based on an amperometric $H_2O_2$ electrode assembled with the enzyme, *L*-lysine oxidase, immobilized on a polymer support placed in intimate contact with the platinum electrode.

The reaction is as follows:

$$L\text{-lysine} + O_2 + H_2O \rightarrow \alpha\text{-keto-}\mathcal{E}\text{-aminocaproate} + NH_3 + H_2O_2$$

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The production of $H_2O_2$ is detected by the platinum electrode held at 650 mV applied potential *vs.* a silver/silver chloride cathode. The output current is correlated to the concentration of lysine in the sample. The use of blocking membranes eliminated all electrochemical and enzymatic interferences. The detection limit ($5 \times 10^{-7} M$) was two orders of magnitude lower than the $O_2$ probe used in previous work.[10]

## EXPERIMENTAL

### *Reagents and materials*

*L*-lysine α-oxidase from *Trichoderma viride* (EC 1.4.3.14) lyophilized powder, 45 U/mg, was obtained from Yamasa Shoyu Co. Tokyo, Japan.

*L*-lysine monohydrochloride (*puriss. p.a.*) was from Fluka (Germany). Other amino acids tested were from Sigma Chemical Co. (St Louis, MO). All other reagents of pure grade were from Farmitalia C. Erba (Milan, Italy).

Five samples of feed (dietetic preparations for laboratory animals) were a gift from ENEA, two samples of feed for rabbits were obtained from a local grocery store.

The Immobilon-AV affinity membrane, 0.65-μ pore size, 125 μm thick was from Millipore (Bedford, MA).

Cellulose acetate membrane, with an approximate molecular weight cut-off of 100, was prepared in our laboratory. The procedure is reported in the literature.[12]

Polycarbonate membrane (0.03-μm pore size) 6 μm thick was from Nucleopore, Pleasanton, CA.

### *Apparatus*

For batch analysis the probe used was a hydrogen peroxide sensor from Universal Sensors Inc. Metaire, LA.

For FIA and flow through analysis we used a wall-jet cell model 656 electrochemical detector from Metrohm, Herisau, Switzerland. This cell was assembled with a platinum working electrode (1.6 mm diameter) (Model MF 2013) from BAS, Lafayette, IN.

Steady state and transient currents were measured with an Amperometric Biosensor Detector ABD from Universal Sensor, Metaire, LA and recorded with an AMEL model 868 recorder, Milan, Italy.

The peristaltic pump was a Minipuls 3 from Gilson, France. For the FIA technique a HPLC Rheodyne valve model 7125 with a closed loop of 20 μl was used.

### *Electrochemical biosensor and biocell assembling*

The biosensor probe consists of a platinum electrode polarized at +650 mV *vs.* a built in silver/silver chloride reference electrode. This probe was assembled by placing on an inverted electrode jacket in the given order the following membranes: cellulose acetate which protects the platinum electrode from electrochemical interferences, [13] the enzyme membrane and a polycarbonate membrane which protects the enzyme from proteins or bacteria. These membranes were then secured with an O-ring. The electrode jacket was filled with a solution of potassium chloride 0.1*M*, then the electrode was inserted into the jacket and screwed down until the tip of the platinum was firmly in contact with the membranes.

The wall jet cell was assembled covering the working electrode with the same membranes. Then the electrode was firmly secured into the cell which was connected to the flow system.

### *Procedures*

Lysine oxidase was immobilized on the Immobilon membrane according to the following procedure: for a single membrane of 1 cm diameter, 200 μg of dry enzyme were placed on the Immobilon membrane, 20 μl of 0.1*M* phosphate buffer pH 7.0 were added, the resulting mixture was homogeneously spread-out on the membrane with a glass stick. The membrane was air-dried for about 1 hr and then kept immersed in the same buffer until ready to use. When the bovine serum albumine (BSA)/glutaraldehyde immobilization on Immobilon was performed we used the procedure described by Villarta *et al.*[14]

### *Batch procedure*

The lysine probe was immersed in 10 ml of stirred phosphate buffer solution (PBS) and then allowed to equilibrate. Aliquots of standard *L*-lysine solutions were then injected into the buffer and the current change recorded.

### *Flow through procedure*

The assembled probe was inserted in the electrochemical wall-jet cell. The buffer was passed through the flow system and a current baseline recorded.

Then lysine standard solutions prepared in the same buffer were introduced into the cell and the current change recorded.

*FIA*

A closed 20-$\mu$l loop of a HPLC valve previously connected in series to the flow system was filled with the analyzing solution, then after the initial steady-state current was recorded, the solution was injected into the flow stream and a transient current variation recorded.

*Feeds*

When feeds were analyzed, samples were first ground to pass through 0.75-mm diameter openings and then mixed thoroughly by tumbling. Then 300-mg samples were weighed and transferred into test tubes. Then 10 ml of 6*N* HCl was added and heated at 120 ± 5° for 24 hr. The hydrolyzate was filtered and adjusted with dry NaOH and 1*M* NaOH to pH values between 6 and 7.5. The solution was then centrifuged, and appropriately diluted with buffer in order to have a response which fits within the calibration curve.

Analysis of lysine by HPLC was carried out using a C 18 reversed phase column and a solvent mixture consisting of acetonitrile and 100 m*M* sodium acetate pH 7.2 pumped at 0.8 ml/min.

## RESULTS AND DISCUSSION

The lysine probe was first used in batch analysis. To select the best immobilization procedure and the optimum response, we assembled our probe using different protective membranes and different immobilization procedures. In previous work[14] it has been demonstrated that an enzyme immobilized on an Immobilon pre-activated membrane has higher specific activity if the immobilization is carried out by adding BSA and glutaraldehyde on the Immobilon membrane.

The same approach has been used in this work. Figure 1 shows calibration curves obtained using the enzyme lysine oxidase immobilized on Immobilon in the presence and absence of BSA and glutaraldehyde, and using polycarbonate membranes of different porosity.

The best response was attained using the BSA/glutaraldehyde procedure on Immobilon and a 0.8-$\mu$m polycarbonate membrane. This result was expected because the polycarbonate membrane has the highest porosity and the

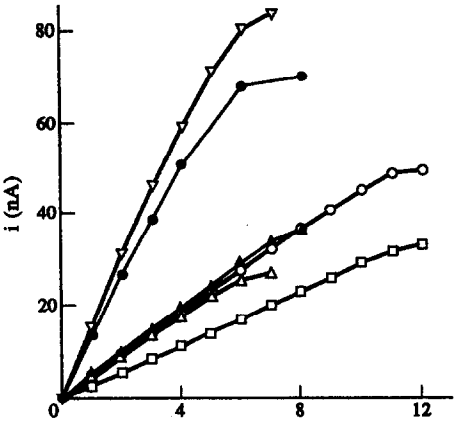


Fig. 1. Lysine calibration curves using two different immobilization procedures and protective polycarbonate membranes with different porosity. ▽ BSA/glutaraldehyde on Immobilon and 0.8-$\mu$m polycarbonate; ● Immobilon only and 0.8-$\mu$m polycarbonate; ○ BSA/glutaraldehyde on Immobilon and 0.03-$\mu$m polycarbonate; ▲ BSA/glutaraldehyde on Immobilon and 0.05-$\mu$m polycarbonate; △ Immobilon only and 0.05-$\mu$m polycarbonate; □ Immobilon only and 0.03-$\mu$m polycarbonate. Buffer phosphate 0.1*M* pH 7.0 $T = 25°$.

immobilization procedure was optimized to have a higher amount of enzyme. However the linearity reached for this immobilization was up to $5 \times 10^{-4}M$ lysine. Using a 0.03-$\mu$m polycarbonate membrane the sensitivity of the probe decreased because of slower diffusion of the substrate through the membrane pores but better linearity was attained. This phenomenon, already observed for other substrates in previous work,[15] allowed us to detect lysine up to $10^{-3}M$.

The enzyme immobilized on Immobilon without BSA/glutaraldehyde gave the same linearity but less sensitivity.

Since our goal was to use this sensor for the analysis of lysine in feeds, we selected the probe assembled with the enzyme immobilized on Immobilon with BSA/glutaraldehyde procedure and with the 0.03-$\mu$m polycarbonate. The last membrane was used to protect the enzyme from possible interferences in the hydrolized matrix, and to obtain an extended linear range of the probe to allow the analysis of a wider range of concentrations. Calibration curves for Fig. 1 were obtained in pH 7.0 phosphate buffer. This pH was previously selected by studying the probe response *vs.* pH with lysine injections of $10^{-4}M$ in a solution containing an appropriate buffer.

Figure 2 shows the current response of our probe used in different buffers for different pHs.

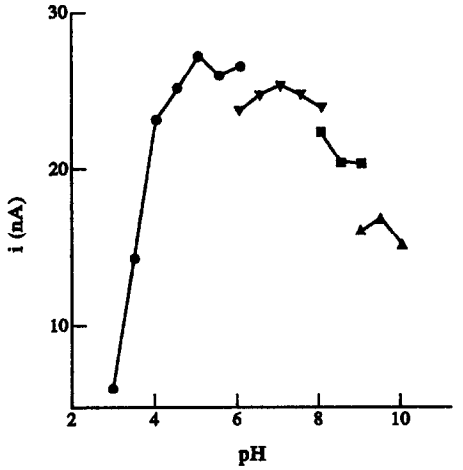


Fig. 2. Effect of pH on lysine oxidase activity. The enzyme activity was measured in the following buffers: ● citrate; ▼ phosphate; ■ tris; ▲ borax, $T = 25°$.

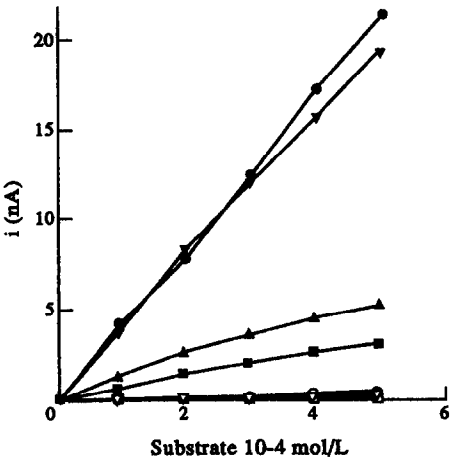


Fig. 4. Relative lysine oxidase activity toward some substrates pH 7.0 phosphate buffer $T = 25°$. ● lysine; ▼ lysine after the analysis of other aminoacids. ▲ ornithine; ■ arginine; ○ tyrosine; □ phenylalanine; ▽ histidine.

The best response was obtained between 5 and 7.5 which enabled use of our probe over a large pH range and in different buffers. For our study, phosphate buffer pH 7.0.

Temperature studies were carried out in the range 15–40°. By increasing the temperature, an increase in the probe response was observed. This effect is due to two factors; the increase in enzyme activity and the increased rate of diffusion substrates through the membrane pores. However, for temperatures up to 30° the current noise also increased, resulting in a lower current/noise signal output.

The best compromise was reached at 25° and this temperature was selected for further experiments.

A calibration curve for lysine after these parameters had been optimized is shown in Fig. 3. The detection limit was $5 \times 10^{-7}M$ with a linearity up to $10^{-3}M$. The apparent Michaelis Constant ($K$m) was $2 \times 10^{-3}$ which is almost 2 orders of magnitude higher than that reported in the literature for the free enzyme (0.04 m$M$).[16]

The reproducibility of our probe was tested by running several calibration curves.

Five consecutive calibration curves gave a maximum variation of 5% over the lysine concentration range $10^{-5}$–$10^{-3}M$.

The catalysis of this immobilized enzyme to oxidize various $L$-aminoacids as $L$-lysine derivatives and amines was investigated. Figure 4 shows calibration curves of lysine, and other aminoacids which gave a detectable response. The major interferent was ornithine followed by

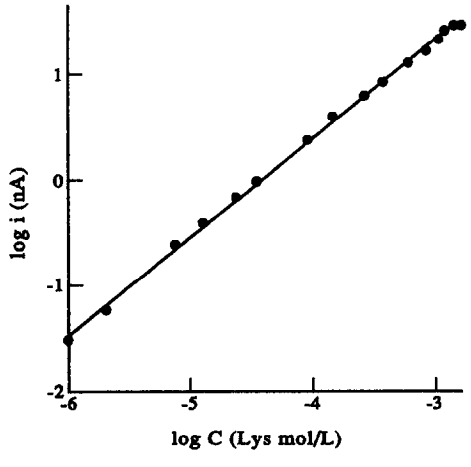


Fig. 3. Lysine calibration curve phosphate buffer 0.1$M$; $T = 25°$; immobilization: BSA/GLU on Immobilon.

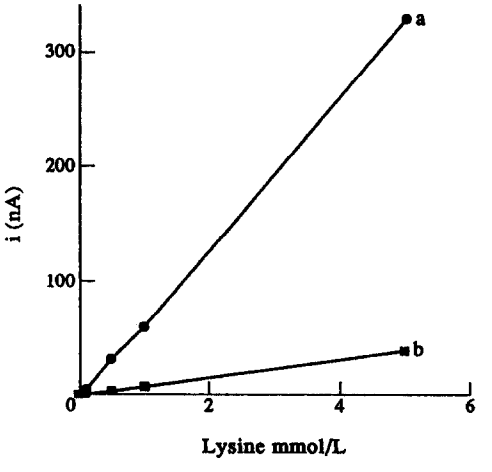


Fig. 5. Flow-through (a) and flow injection (b) detection of lysine. Sample carrier phosphate buffer 0.1$M$ pH 7.0 $T = 25°$.

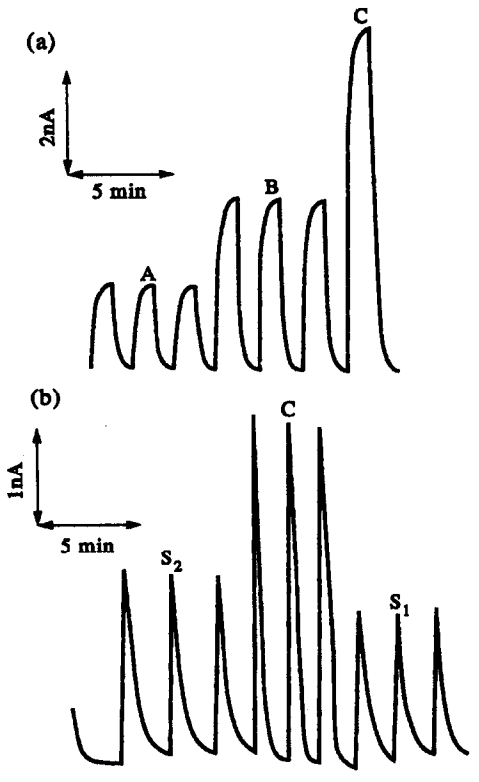


Fig. 6. Reproducibility and response time of the bioprobe in flow through analysis and FIA a = flow through: lysine concentration in standard solution. A = $5 \times 10^{-5}M$; B = $10^{-4}M$; C = $2 \times 10^{-4}M$; b = FIA; $S_1$ and $S_2$ foodstuff samples. C = lysine standard $5 \times 10^{-4}M$.

arginine. The effects of tyrosine, phenylalanine and histidine were negligible. Other aminoacids did not give any appreciable response. A calibration curve for lysine was run after measurements of all interferences were studied. Complete signal recovery was attained.

The lifetime of our sensor was investigated by studying the decrease of the enzyme activity with time.

The probe lost 70% of its original activity in 10 days, then it remained constant for more than 90 days. This decrease in enzyme activity did not pose any particular problem to probe performance. In fact analyses of lysine could be performed in the range $10^{-5}$–$10^{-3}M$ with a detection limit of $5 \times 10^{-6}M$. These concentrations largely cover the range for feed analyses.

The operational time of our probe was more than 300 analyses of lysine. The response time was 2 min with 90% response in less than 1 min.

In order to make this method suitable for continuous flow analysis we used a wall-jet electrochemical cell with a lysine probe, assembled as previously described, inserted into the cell. Calibration curves of lysine were optimized by varying the flow rate to select the best linear range and the lower and upper detection limits of the analysis. The best results were attained at a flow rate of 0.2 ml/min, compromise between the probe sensitivity and stability and the time of analysis.

Analysis of lysine by FIA was carried out at the same flow rate used for continuous flow analysis. In this case an injection valve with a closed loop of a 20-$\mu$l sample was placed between the peristaltic pump and the electrochemical cell. Results are reported in Fig. 5. As is shown in this figure, the flow analysis is much more sensitive than FIA but despite this, the response time of FIA is less than 30 sec and the reproducibility is higher. However the sensitivity of the two methods was good enough to carry out analysis of lysine in foodstuff.

Figure 6 shows the reproducibility of the sensor both in flow analysis and FIA.

*Feed analysis*

Table 1 reports the determination of lysine in some products used to feed animals. The analysis was carried out by continuous flow and by FIA. Both flow-through and FIA methods correlated well with the HPLC procedure with a relative error varying between 1 and 12%. Only one sample gave an error of 17% in flow/HPLC correlation and 13% in FIA/HPLC correlation but gave only 3% when both the amperometric

Table 1. Lysine concentration (% w/w) in foodstuff determined with the biosensor method (continuous flow and FIA) and by HPLC

| Sample | Flow | FIA | HPLC | Relative error ($E$%) | | |
|---|---|---|---|---|---|---|
| | | | | Flow/HPLC | FIA/HPLC | Flow/FIA |
| 1 | 0.171 | 0.164 | 0.167 | 2.4 | 1.8 | 4.1 |
| 2 | 0.198 | 0.189 | 0.191 | 3.7 | 1.0 | 4.6 |
| 3 | 0.128 | 0.115 | 0.127 | 0.8 | 9.4 | 10.2 |
| 4 | 0.089 | 0.086 | 0.076 | 17.2 | 13.2 | 3.4 |
| 5 | 0.123 | 0.112 | 0.110 | 11.8 | 1.8 | 8.9 |
| 6 | 0.074 | 0.071 | 0.066 | 12.1 | 7.6 | 4.1 |
| 7 | 0.061 | 0.055 | 0.058 | 5.2 | 5.2 | 9.8 |

procedures were compared. Values obtained with the three methods were the mean of three determinations. Each sample was determined within 2 min, in flow, and 30 sec in FIA.

During the analysis of feeds the required washing time was longer than that measured during calibration with the buffer solution. This is probably due to the effect of some compounds present in the matrix which adhere to the polycarbonate membrane and require a longer washing time. Using the buffer solution as a washing solution between foodstuff samples, the time required to recover the initial current background was 3 min for flow through analysis and 2 min for FIA.

This allows a sampling rate of 12 samples/hr in flow through analysis and 20 samples/hr in FIA.

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