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Aspartate analysis in formulations using a new enzyme sensor*

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Abstract: A biosensor has been developed for the purpose of directly analysing aspartate in pharmaceutical formulations and aspartame in sweeteners. This biosensor consists of an ammonia-sensitive gas-diffusion electrode and the enzyme L-aspartase immobilized by means of polyazetidine on a dialysis membrane.

Keywords: *Aspartate; aspartame; analysis; biosensor.*

Introduction

Aspartic acid is a non-essential amino acid that is, nevertheless, required by the majority of living organisms [1]. It plays an important role also in the neurological field, as it is present in micromolar concentrations in the extracellular brain fluid [2].

In the pharmaceutical field, aspartic acid is used to prepare specific drugs used to treat the deficiency of certain ions, such as potassium and magnesium, which can be returned to the organism in the form of aspartic acid salts [3]. In the food industry it is present in several dietetic products as a constituent of the dipeptide aspartame (i.e. the methyl ester of aspartylphenylalanine).

The analytical techniques developed for aspartic acid determination are the same as those commonly used to assay other amino acids, e.g. chromatography. The currently most commonly used separation technique is ion exchange chromatography [4–6]. Other widely used methods are gas chromatography [7] and HPLC techniques [8, 9], as well as thin layer chromatography [10, 11], colorimetric [1, 12] or microbiological methods [13] are also used. Moreover, as for amino acids in general, aspartic acid can be determined using spectrophotometric enzymatic detection methods [14], or else by means of electroenzymatic sensors [15–17]. The present paper describes how a biosensor suitable for aspartate determination was developed.

We recently obtained good urea and creatinine biosensors [18, 19] by modifying the response of a classical potentiometric gas-diffusion electrode for ammonia (indicator electrode) using a polymeric membrane with a suitable enzyme immobilized in it (enzyme cellulose triacetate membrane).

The same technique, but a different enzyme immobilization method, has now been applied by making use of the L-aspartase enzyme to obtain a simple biosensor for L-aspartate. The sensor has been tested in the analysis of several commercial drugs containing aspartate, or sweeteners containing aspartame.

Experimental

Reagents

L-aspartase (EC 4.3.1.1.) (extracted from the bacterium Hafnia alvei) and the dialysis membrane (cat. D-9777) were supplied by the Sigma Chemical Company (St Louis, MO, USA).

The gas-permeable membrane (Celgard 2400 Microporous Membrane) was supplied by the Hoechst Celanese Corporation (NC, USA). The inner electrolytic solution for the ammonia electrode was supplied by Ingold (Frankfurt/Main, Germany) art. 152301000. The polyazetidine prepolymer (PAP) solution

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(Hercules Polycup 172; 12% w/w in water) was supplied by Hercules Inc. (Wilmington, DE, USA).

The L-aspartic, D-aspartic and D,L-aspartic amino acids, L-leucine, L-alanine, L-methionine, L-cysteine, L-phenylalanine, L-valine, L-asparagine, L-glutamine, aspartame, L-glutamic acid and cellulose triacetate (TAC) were purchased from Fluka AG (Buchs, Switzerland).

The phosphate, Tris and all the other reagents used (analytical grade) were supplied by Farmitalia Carlo Erba (Milan, Italy).

Apparatus

Measurements were performed using an Amel model 631 potentiometer, coupled to an Amel (Milan, Italy) model 686 recorder. The potentiometric gas-diffusion ammonia electrode was an Ingold art. 152303000, the original membrane of which was replaced with a Celgard Microporous membrane. The temperature was maintained constant by a Julabo 5B thermostat. The glass thermostatted cell, 10-ml capacity, was obtained from Marbaglass (Rome, Italy). The solution in the cell was maintained under stirring using a magnetic microstirrer supplied by Velp Scientifica (Italy).

Immobilization of the enzyme

The enzyme was immobilized on the dialysis membrane using a mixed method of immobilization (i.e. together chemical and physical) [20]: 2 mg of the aspartase enzyme were mixed with 20 μl of the prepolymer solution (PAP), directly on the dialysis membrane. The membrane was left for 24 h at +4°C and then washed with 0.04 M Tris buffer, pH 7. The result was a block polymer, chemically bonded to itself and with good adhesion to the dialysis membrane. The enzyme membrane thus obtained was superimposed on the gas ammonia electrode. Any enzyme loss was then prevented by covering the head of the sensor with another dialysis membrane and the three membranes were secured to the sensor by a rubber O-ring.

The procedure used to immobilize the enzyme, by entrapment in cellulose triacetate membrane, is the same as that described in detail in a previous paper [21]. Briefly: by dissolving a weighed amount of TAC in formic acid–water (90:10, v/v) under magnetic stirring we obtained a viscose that, when stratified on a glass plate and dipped into distilled water, yielded a gelled polymeric film. By cutting this film into disks, after abundant washing to remove the formic acid completely, we obtained a membrane free of any harmful agent and porous enough to allow the enzyme to diffuse into it freely. After drying, the irreversible contraction of the fibres resulted in entrapment of the enzyme. The enzyme membrane was secured to the sensor by means of a dialysis membrane and of a rubber O-ring.

Lastly, chemical immobilization in a functionalized nylon membrane using carbodiimide was also attempted following the procedure described in a previous paper [22].

Biosensor assembly and principle underlying the method

The indicator electrode used to assemble the biosensor is a commercially available potentiometric gas-diffusion electrode for ammonia determination. It consists essentially of a glass electrode dipping into an aqueous ammonium solution (inner electrode solution). The latter solution is separated from the test solution by a gas-permeable membrane which thus allows ammonia to pass but not the electrolytes (Celgard Microporous membrane) (Fig. 1).

The gas-diffusion potentiometric electrode (indicator electrode) thus described is modified

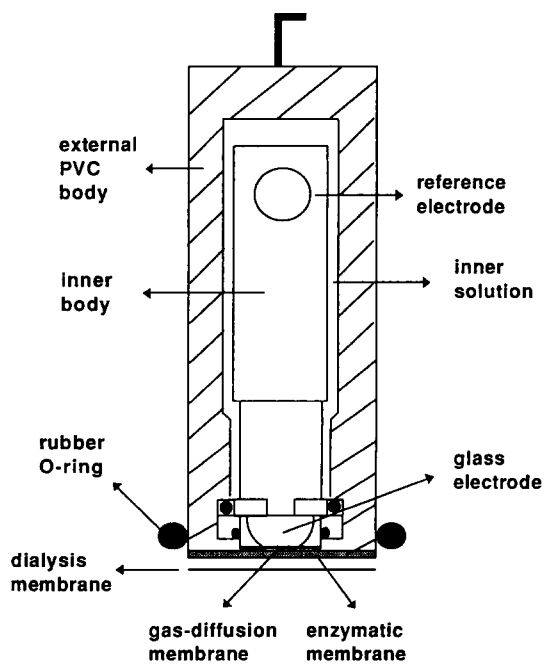

Figure 1
Scheme of the L-aspartate biosensor.

by superimposing on the gas-permeable membrane another membrane containing an enzyme (L-aspartase) which catalyses a reaction during which ammonia is produced.

$$\text{L-aspartate} \xrightarrow{\text{L-aspartase}} \text{fumarate} + NH_3$$

The aspartate assay can be performed by relating the substrate concentration to that of the ammonia produced during the reaction.

The measurements were carried out in steady-state conditions, in a 10-ml glass cell, thermostatted by means of forced water circulation, under magnetic stirring.

Sample analysis

The present work involved using the biosensor to analyse several drugs containing aspartate and several sweeteners containing aspartame.

The pharmaceutical formulations containing aspartate and the sweeteners containing aspartame analysed were all commercial products. Their composition and the nominal value of the percentage content of the substances are shown in Table 1.

The samples examined were available in several different pharmaceutical forms (vials, tablets, powders). Before analysis with the enzymatic sensor, the drugs in vial form were suitably diluted. The tablets and powders (both drugs and sweeteners) were ground up, weighed and then solubilized. At the end of the various treatments, the concentration of the aspartate or aspartame in the solutions tested was about 0.1 M so that the final concentration in the measurement cell fell in the linearity range of the method.

The biosensor was immersed in a cell thermostatted at 30°C containing 5 ml of Tris

Table 1
Percentage by weight composition of the pharmaceutical products and sweeteners analysed by the enzyme sensor. Nominal values as supplied by manufacturing firms

Drug no. and its pharmaceutical form	Composition	Content (% w/w)
1 (vials)*	D-L aspartate (potassium salt)	17.11
	Distilled water	82.89
2 (tablets)*	D-L aspartate (potassium salt)	33.8
	D-L aspartate (magnesium salt)	22.8
	Gelatine	2.0
	Starch	0.8
	Magnesium stearate	1.5
	Cellulose acetate phthalate	1.6
	Cellulose diethylphthalate	0.6
	Calcium carbonate	4.0
	Talc	1.6
	Silica	0.4
	Titanium dioxyde	0.8
	Polyethylene glycol 6000	1.4
	Polyvinyl pyrrolidone	0.18
	Potassium monophosphate	0.04
	Glucose	0.35
	Mastic rubber	0.03
	White wax	0.02
	Sucrose	28.08
3 (powder)*	L-aspartate (potassium salt hemihydrate)	4.5 (2.3)‡
	L-aspartate (magnesium salt tetrahydrate)	4.5 (3.6)‡
	Tartaric acid	7.5
	Anhydrous citric acid	1.0
	Natural orange flavour	0.025
	Sodium bicarbonate	4.0
	Lyophilized orange	10.0
	Sucrose	68.25
4 (tablets)†	Aspartame	50
	Excipients	50
5 (tablets)†	Aspartame	50
	Excipients	50

* Pharmaceutical preparation.
† Sweetener.
‡ % of anhydrous salt.

buffer 0.1 M at pH 8.5 and magnesium chloride 1.4×10^{-4} M, and the signal allowed to stabilize. The sample solution was then added (25–200 µl, about 0.1 M). The potentiometric signal was read off after about 3 minutes. The potentiometric value obtained was then inserted in the calibration straight line obtained using the method described above. In this way it was possible to determine the concentration of aspartate in the sample.

The calibration curve was obtained under the same conditions by successively adding standard solution (0.1 M) of L-aspartate (or D,L-aspartate, or aspartame) to 5 ml of the buffer solution described above.

Results

First, the biosensor obtained by using polyazetidine to immobilize the enzyme L-aspartase on the dialysis membrane, was characterized.

Working conditions were optimized by observing sensor response as a function of pH and the nature of the buffer (Fig. 2). The response was also investigated as a function of the temperature (Fig. 3). Lastly, sensor response time was measured (Fig. 4). The results show that optimal working conditions are as follows: buffer = Tris 0.1 M, pH 8.5, response time = 3 min, temperature = 30°C.

Table 2 gives the main analytical data obtained using the biosensor with standard solutions of L-aspartate and with the enzyme aspartase immobilized on a dialysis membrane by means of polyazetidine. Table 3 shows the repeatability of the calibration curve data obtained using this biosensor in standard L-aspartate solutions.

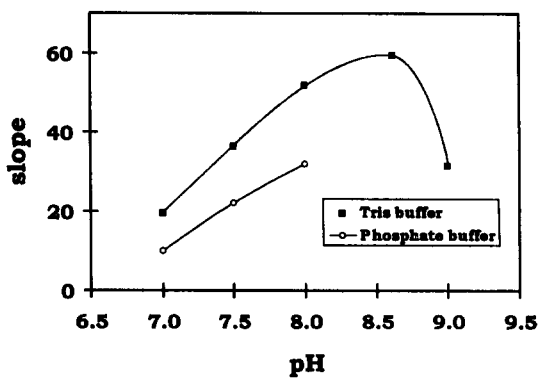

Figure 2
Trend of the biosensor response as a function of the pH and buffer nature.

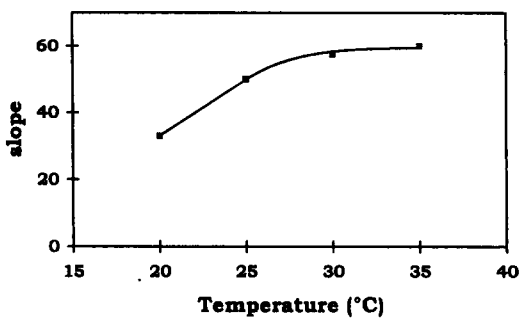

Figure 3
Trend of the biosensor response as a function of the temperature.

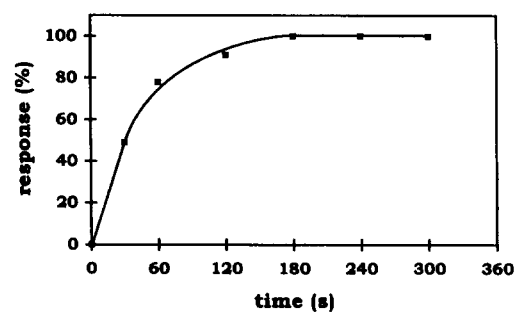

Figure 4
Percentage response of the biosensor as a function of the response time.

Table 2
Main analytical data in the analysis of L-aspartate by enzyme sensor with the enzyme L-aspartase immobilized (a) in dialysis membrane using polyazetidine, (b) in cellulose triacetate membrane

	(a)	(b)
Lifetime (days)	≥20	≈1
Response time (min)	3	3
Linearity range (M)	1.9×10^{-3}–2.4×10^{-2}	1.9×10^{-3}–2.4×10^{-2}
Minimum detection limit (M)	4.9×10^{-4}	1.0×10^{-3}
Precision on standard solutions (RSD%)	3.8	7.0
Inaccuracy on standard solutions (% values)	−6.1–+6.8	−5.8–+7.2
Calibration curve (E = mV; c = M)	$E = -60.0 \log c - 140.1$	$E = -31.8 \log c - 90.6$
Correlation coefficient, r	−0.9902	−0.9748

Table 3
Reproducibility data of the calibration graph, by enzyme sensor, in standard solutions of L-aspartate. L-aspartase immobilized (a) in dialysis membrane using polyazetidine, (b) in cellulose triacetate membrane

Calibration no.	Linearity range (M)	Slope (ΔmV/Δlog$c$)	Intercept (ΔmV)	Correlation coefficient (r)
(a)				
1	1.9×10^{-3}–2.4×10^{-2}	−61.8	−143.6	−0.9924
2	1.9×10^{-3}–2.4×10^{-2}	−57.1	−139.1	−0.9938
3	1.9×10^{-3}–2.4×10^{-2}	−60.7	−136.7	−0.9846
4	1.9×10^{-3}–2.4×10^{-2}	−60.4	−141.1	−0.9900
Mean	1.9×10^{-3}–2.4×10^{-2}	−60.0 (RSD% = 2.9)	−140.1 (RSD% = 1.8)	−0.9902
(b)				
1	1.9×10^{-3}–2.4×10^{-2}	−35.6	−89.2	−0.9830
2	1.9×10^{-3}–2.4×10^{-2}	−34.3	−87.3	−0.9821
3	1.9×10^{-3}–2.4×10^{-2}	−31.3	−79.4	−0.9521
4	1.9×10^{-3}–2.4×10^{-2}	−29.8	−77.1	−0.9821
Mean	1.9×10^{-3}–2.4×10^{-2}	−32.7 (RSD% = 7.0)	−83.2 (RSD% = 6.1)	−0.9748

A study was also made of the feasibility of physically immobilizing the enzyme in a cellulose triacetate membrane using a method previously developed in this laboratory [21]. The analytical characterization of the sensor was performed under the same experimental conditions as were used for the biosensor obtained by immobilizing the enzyme as described above. The main analytical data thus obtained are shown in Table 2 and their repeatability in Table 3. The latter tables show that the response obtained is much weaker than when the sensor is obtained by immobilizing the enzyme by means of polyazetidine.

In Table 4 a comparison is made between the relative activity of the latter type of sensor towards L-aspartate assumed to be 100% and that displayed by the same sensor towards other more common L-amino acids as well as towards D-aspartate.

Lastly, in Fig. 5 the response of the sensor obtained by immobilizing the enzyme with polyazetidine is plotted against time starting from the day of assembly.

In order to use this biosensor to analyse samples of commercial drugs containing L-aspartate, D,L-aspartate, or sweeteners containing aspartame, it was necessary to construct calibration curves also for the D,L-aspartate and the aspartame. Table 5 shows the main analytical data referring to the calibration curves for the D,L-aspartate and the aspartame. The repeatability of the same calibration curves is set out in Table 6.

Only the biosensor obtained by immobilizing the enzyme on a dialysis membrane by means of polyazetidine, i.e. the biosensor found to be the most efficient, was used in the analysis of real samples. The samples tested (drugs and sweeteners listed in Table 1) received no pretreatment before being analysed. The precision

Table 4
Interferences in the analysis of L-aspartate by the L-aspartase biosensor

Substrate	Relative activity % to tested species*
L-aspartate	100
L-alanine	0
L-leucine	5
L-phenylalanine	0
L-valine	0
L-glutamic acid	0
L-asparagine	98
L-glutamine	57
D-aspartate	0
NH_3	100

* As percentage value of the slope of calibration graph, obtained using each of the species by interference activity as substrate, referred to the slope of the L-aspartate calibration graph, assumed to be 100%.

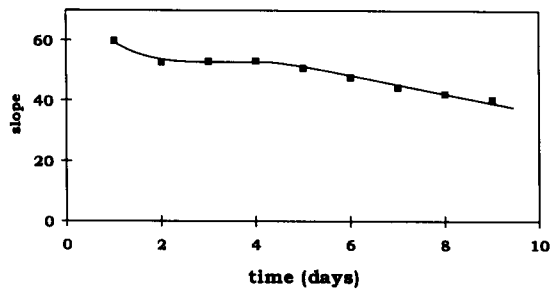

Figure 5
Lifetime and trend of the biosensor response as a function of the elapsed time from its preparation.

Table 5
Main analytical data for the analysis of D,L-aspartate and aspartame by enzyme sensor with the enzyme L-aspartase immobilized in dialysis membrane using polyazetidine

	D,L-aspartate	Aspartame
Lifetime (days)	≥20	≥20
Response time (min)	3	3
Linearity range (M)	3.8×10^{-3}–2.4×10^{-2}	3.8×10^{-3}–2.6×10^{-2}
Minimum detection limit (M)	1.9×10^{-3}	2.6×10^{-3}
Precision on standard solutions (RSD%)	3.6	4.2
Inaccuracy on standard solutions (% values)	−5.4–+5.8	−5.3–+5.7
Calibration curve (E = mV; c = M)	$E = -21.7 \log c - 56.2$	$E = -46.2 \log c - 116.8$
Correlation coefficient, r	−0.9935	−0.9772

Table 6
Reproducibility of the calibration graph, by enzyme sensor, in standard solutions of (a) D,L-aspartate and (b) aspartame. L-aspartase immobilized in dialysis membrane using polyazetidine

Calibration no.	Linearity range (M)	Slope (ΔmV/Δlog$c$)	Intercept (ΔmV)	Correlation coefficient (r)
(a)				
1	3.8×10^{-3}–2.4×10^{-2}	−21.9	−59.1	−0.9976
2	3.8×10^{-3}–2.4×10^{-2}	−21.6	−55.3	−0.9958
3	3.8×10^{-3}–2.4×10^{-2}	−21.5	−54.3	−0.9872
Mean	3.8×10^{-3}–2.4×10^{-2}	−21.7 (RSD% = 0.8)	−56.2 (RSD% = 3.7)	−0.9935
(b)				
1	3.8×10^{-3}–2.6×10^{-2}	−47.5	−117.8	−0.9713
2	3.8×10^{-3}–2.6×10^{-2}	−44.7	−116.2	−0.9785
3	3.8×10^{-3}–2.6×10^{-2}	−46.5	−116.8	−0.9818
Mean	3.8×10^{-3}–2.6×10^{-2}	−46.2 (RSD% = 2.5)	−116.9 (RSD% = 0.6)	−0.9772

Table 7
Repeatability of L-aspartate or aspartame determination, in commercial drugs and sweeteners, using the enzyme sensor and comparison of found results by nominal values supplied by manufacturing firms

Drug no. and its pharmaceutical form	Nominal value (as % by weight) (a)	Value found by enzyme sensor (as % by weight)	Mean value (as % by weight) (b)	RSD%	$\frac{b-a}{a}$ (%)
1 (vials)*	17.1	16.5 16.8 17.4	16.8	3.2	−1.8
2 (tablets)*	56.6	54.2 55.6 55.6	55.1	1.2	−2.6
3 (powder)*	5.9	5.6 6.1 6.5	6.1	6.1	+3.4
4 (tablets)†	50.0	55.5 49.7 50.2	51.7	5.0	+3.5
5 (tablets)†	50.0	47.0 48.0 53.5	49.5	5.7	−1.0

*Pharmaceutical preparation.
†Sweetener.

Table 8
Recovery of aspartate or aspartame, by the standard addition method, in pharmaceutical formulations or sweeteners. Reported values are the final concentrations after proper dilution

Sample	Found value (mM)	Standard added (mM)	Total found value (mM)	Recovery %
	4.4	4.0	8.6	102.4
1 (vials)*	4.4	8.0	12.4	100.0
	4.4	12.0	16.1	98.2
	3.9	4.0	8.2	103.4
3 (powder)*	3.9	8.0	11.7	98.3
	3.9	12.0	16.3	102.5
	19.2	4.0	25.1	108.1
4 (tablet)†	19.2	8.0	30.2	111.0
	19.2	12.0	30.7	98.4

* Pharmaceutical preparation.
† Sweetener.

of aspartate determination in the various commercial drugs and of aspartame in the typical commercial sweeteners is listed in Table 7. Table 8 sets out the yields obtained using the standard additions method for some drug and sweetener samples. The agreement between concentration values found using the biosensor and the nominal concentration values declared by the manufacturers of the commercial samples tested is also shown in Table 7.

Discussion

In order to respect the actual chronological order in which the tests were carried out, the construction of the biosensor used to assay L-aspartic acid using the enzyme L-aspartase was begun by first physically immobilizing the enzyme (entrapment in cellulose triacetate membrane), a method that has proved highly satisfactory with other enzymic systems (see Introduction) [18, 21]. A chemical method of immobilization was then tried (imobilization in functionalized nylon membrane) [22]. However, the results in the latter case were extremely poor, even though on other occasions [22, 23] this type of immobilization proved to be effective. Lastly, a "mixed" type of immobilization (i.e. both chemical and physical) was tried using polyazetidine as reagent and a dialysis membrane as support. Tests (Table 2) clearly showed that the best response towards L-aspartate is actually obtained using the "mixed" method. By immobilizing the L-aspartase in a cellulose triacetate membrane instead of with polyazetidine, biosensor sensitivity is reduced considerably and measurement repeatability is low, while the linearity range remains more or less constant (Table 2). Furthermore, it was not possible to monitor the working life of the sensor with the cellulose triacetate enzymatic membrane as enzyme activity decreased dramatically as early as the second day. On the other hand, the curve in Fig. 5 shows how, using polyazetidine immobilization, biosensor response, and thus membrane activity, after a slight decline between day one and day two, remains practically unchanged during the subsequent 4 days of daily biosensor use. At the end of this period, a very slow, practically constant, decline is observed in enzyme activity and the sensor can be used for up to 3–4 weeks. After 10 days or so, sensor response was found to have decreased by only about one third of its initial value.

The next step was to optimize the operating parameters (pH, choice of buffer, temperature and evaluation of response time). The optimal pH value was determined by using two different buffers (phosphate and Tris) and carrying out tests over the pH range 7.0–9.0. The tests indicated that the best sensor response (Fig. 2) is obtained using Tris buffer at pH 8.5.

The other parameter examined was the working temperature: sensor sensitivity and thus also enzymatic activity increases up to a temperature of about 30°C, after which it remains practically constant from 30 to 35°C (Fig. 3). The lower temperature (30°C) was thus selected as it corresponded to almost maximum activity and the fewest drawbacks. In fact, the higher the temperature the greater the number of operating problems, as the sensor signal tends to become unstable, which

naturally leads to less repeatable measurements.

Investigation of the response time indicates that the biosensor delivers the highest response after about 3 min, after which it remains constant for a longer period of time (Fig. 4).

In addition, all the tests were carried out in the presence of magnesium chloride. It is widely reported in the literature that magnesium acts as a metallic cofactor, thus increasing the catalytic activity of the L-aspartase enzyme [24].

Biosensor response was measured also for several possible interfering substances. Table 4 shows that, in addition to that of course due to ammonia, the greatest interference is due to L-asparagine and L-glutamine. While in the case of L-asparagine the response is almost certainly due to the enzymatic reaction, in the case of L-glutamine a sensor response is observed also in the absence of the enzyme L-aspartase. The underlying cause is thus to be sought in the reaction of glutamine hydrolysis with ammonia release which occurs in an alkaline environment.

The biosensor obtained by immobilizing the enzyme L-aspartase by means of polyazetidine was also found to be highly sensitive (the slope is slightly overNernstian), differently from what was observed for the biosensor obtained by immobilizing the enzyme in TAC (strongly underNernstian) (Table 2). Measurements are also highly repeatable over a comparatively narrow linearity range, which is, however, wide enough to allow satisfactory application to real samples (Table 3).

The L-aspartase sensor was used to test commercial drugs and sweeteners respectively containing L-aspartate, D,L-aspartate, or aspartame (Table 1). The good repeatability of the measurement of the aspartate contained in drugs and a satisfactory repeatability in analysing the aspartame contained in sweeteners is apparent from Table 7. For some of the samples examined accuracy tests using the standard additions method were also carried out (Table 8). For the test samples a comparison was also made between the measured aspartate or aspartame concentrations and the values declared by the manufacturing firms (Table 7). The positive results obtained in all these tests indicate that the biosensor developed by us is certainly capable of providing sufficiently accurate results in the analysis of samples of commercial drugs or sweeteners.

Conclusions

Several different sensors for aspartate analysis, or L-aspartase enzyme activity checking, have been prepared by various workers in recent years [16, 17, 25, 26]. In some cases the L-aspartase enzyme [16, 17] was used, in one case a bacterial membrane [25], and in other cases a dual enzyme electrode [26, 27]. The latter sensors and bacterial probe are undoubtedly very complex to assemble and employ, while those based solely on the immobilized L-asparatase enzyme are generally easier to assemble and handle. The biosensor described herein belongs to the latter type of biosensor. As we have seen, immobilization by means of polyazetidine, as well as being very rapid and easy, is also very efficient. There is only a slight loss of enzymatic activity over time, so that the sensor has an effective working life of over three weeks. Indeed, the entire analytical performance of the sensor proposed by us is equivalent to or better than that displayed by similar L-aspartase sensors [16, 17]. In conclusion, there is no lack of amino acid sensors that employ less specific enzymes, e.g. L-aminoacid oxidase [28, 29], or decarboxilase [30], or electrochemical sensors based on liquid or polymeric ion selective membranes described in the literature [30–32]. Indeed, we ourselves six years ago proposed a polymeric membrane ion-selective electrode (ISE) that was responsive to L-aspartate [33]. However, this type of sensor, although having a shorter response time than the enzymatic type, has an extremely narrow selectivity compared with the high specificity of enzymatic sensors. Nevertheless, this property of biosensors is extremely important when performing direct analyses on real samples when good results are aimed for, as in the case of the sensor used by us to test several pharmaceutical formulations and sweeteners.

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References

[1] G.C. Barret, *Chemistry and Biochemistry of the Aminoacids*, pp. 7, 11. Chapman and Hall, London (1985).
[2] H. Benveniste, *J. Neurochem.* **22**, 1667–1679 (1989).

[3] *Ullman's Encyclopedia of Industrial Chemistry*, Vol. 22 (B. Elvers, S. Hawkins, W. Russey and G. Schulz (Eds), pp. 453–458. Weinheim, Germany (1993).
[4] P.B. Hamilton, *Anal. Chem.* **35**, 2055–2064 (1963).
[5] H.M. Lee, M.D. Forde, M.C. Lee and D.J. Bucher, *Anal. Biochem.* **96**, 298–307 (1979).
[6] S. Moore and W.H. Stein, *J. Biol. Chem.* **211**, 907–913 (1954).
[7] C.J. Goh, K.G. Craven, J.R. Lepock and E.B. Dumbróff, *Anal. Biochem.* **163**, 175–181 (1987).
[8] D.W. Aswad, *Anal. Biochem.* **137**, 405–409 (1984).
[9] E.D. Willis, *J. Chromatogr.* **408**, 217–225 (1987).
[10] E.V. Truter, *Thin Film Chromatography*, pp. 132–137. Cleaver-Hume Press Ltd, London (1963).
[11] K. Malek, Z. Deyl and M. Smrz, *J. Chromatogr.* **193**, 421–426 (1980).
[12] M. Li, *Yaown Fenxi Zazhi* **2**, 151–153 (1982).
[13] H. Hithoh, T. Morimoto and I. Chibata, *Anal. Biochem.* **60**, 573–580 (1974).
[14] G.G. Guilbault and E. Hrabankova, *Anal. Letts* **3**, 53–57 (1970).
[15] M. Nanjo and G.G. Guilbault, *Anal. Chim. Acta* **73**, 367–373 (1974).
[16] G. Davis, R.S. Haddad and W.R. Hussein, *Anal. Letts* **20**, 259–274 (1987).
[17] O. Fatibello-Filho, A.A.F. Suleiman and G.G. Guilbault, *Biosensors* **137**, 313–321 (1989).
[18] L. Campanella, M.P. Sammartino and M. Tomassetti, *Analyst* **115**, 827–830 (1990).
[19] L. Campanella, F. Mazzei, M.P. Sammartino and M. Tomassetti, *Bioelectrochem. Bioenerg.* **23**, 195–202 (1990).
[20] L. Campanella, F. Mazzei, C. Morgia, M.P. Sammartino, M. Tomassetti, V. Baroncelli, M. Battilotti, C. Colapicchioni, I. Giannini and F. Porcelli, *Analusis* **16**, 120–124 (1988).
[21] L. Campanella, M. Tomassetti and M.P. Sammartino, *Sensor Actuat.* **16**, 235–245 (1989).
[22] L. Campanella, R. Cocco, M.P. Sammartino and M. Tomassetti, *Sci. Tot. Environ.* **123/124**, 1–16 (1992).
[23] L. Campanella, R. Cocco and M. Tomassetti, *J. Pharm. Biomed. Anal.* **10**, 741–749 (1992).
[24] V.R. Williams and D.J. Lartigue, *Methods in Enzimology* (J.M. Lowenstein Ed.), pp. 354–361. Academic Press, New York (1969).
[25] R.K. Kobos and G.A. Rechnitz, *Anal. Letts* **10**, 751–758 (1977).
[26] R.L. Villarta, G. Palleschi, G.J. Lubrano, A.A. Sulliman and G.G. Guilbault, *Anal. Chim. Acta* **245**, 63–69 (1991).
[27] C.P. Pau and G.A. Rechnitz, *Anal. Chim. Acta* **160**, 141–147 (1984).
[28] R. Ianniello and A.M. Yacynych, *Anal. Chim. Acta* **131**, 123–132 (1981).
[29] R. Ianniello and A.M. Yacynych, *Anal. Chim. Acta* **53**, 2090–2095 (1981).
[30] T. Katsu, T. Kayamoto and J. Fujita, *Anal. Chim. Acta* **239**, 23–27 (1990).
[31] G.M. Sergeev, I.M. Karenman and I.V. Blokhina, *Zhurn. Analitich. Khimii* **35**, 1184–1187 (1980).
[32] T. Shinbo, T. Yamaguchi, K. Nishimura, M. Kikkawa and M. Sugiura, *Anal. Chim. Acta* **193**, 367–371 (1987).
[33] L. Campanella, F. Mazzei, R. Sbrilli and M. Tomassetti, *Ann. Chim.* **79**, 335–353 (1989).