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Electrochemical Analysis on Compounds of the Vitamin B_6 Family Using Glassy Carbon Electrodes

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The electrochemical reduction of vitamin B6 group and related compounds has been performed at pH neutral using glassy carbon electrodes. Irreversible bi- or tetra-electronic processes controlled by diffusion on the top of the wave are observed for these substances by cyclic voltammetry. In most cases the first electron transfer was the rate determining step of the electrode process. In depth electroanalytical behaviour of the most important members of the vitamin-6 family, from a biological point of view, is also presented. Limits of detection (99, 59, 286 and 19 μM respectively), linearity ranges (200-1000, 100-800, 400-1600 and 40-200 μM respectively) precision as RSD(%) (0.45, 4.11, 6.45 and 4.98 % respectively) and recovery (%) values (99.7, 97.7, 104.4 and 98.9) for Pyridoxal, Pyridoxal-5'-Phosphate, Pyridoxamine and Pyridoxic Acid are presented. The use of glassy carbon electrodes for the analysis of urine samples gives an example of the potential use of these for the analysis of biological samples.

Keywords: electrochemistry; vitamin B-6; glassy carbon electrodes; colon-rectal cancer

1. INTRODUCTION

Most of the physical chemical studies on vitamin B_6 have been performed on the natural derivatives of pyridoxine trying to understand their role in amino acid and lipid metabolism. The biological relevance and importance of the vitamin B_6 family is paramount as most of them can be interconverted through redox and phosphorilation reactions, which makes understanding these reactions even more relevant.

Knowing B_6 vitamers concentration in biological fluids or under physiological conditions (pH 7) is proving very important for the control of different serious conditions. The importance of vitamin

B_6 and its role in preventing rectal-colon has been recently highlighted[1-3]. In these studies the incidence of colorectal cancer is inversely associated to the concentration of pyridoxine-5'-phosphate in blood. Similar results were found when the PLP blood concentrations were tested and associated to the frequency of pancreatic[4], lung[5] and gastric[6] and oral or pharyngeal cancers[7]. Normal PLP concentration has also been related to a lower risk to suffer coronary diseases[8] and strokes[9]. Control and quick analysis of the B6 vitamers can be extremely useful for maintaining an accurate control on the evolution of these conditions in patients.

At present, B_6 vitamers have been mostly analysed using HPLC attached to great variety of detectors, the most common being fluorescent[10,11], Diode Arrayf [11] and electrochemical[12] with no[11,12] pre-column[13] or post-column derivatisation steps[10,14]. Other methods used to determine their concentration rely on the use of enzymatic methods[15] and more recently using GC-MS and LC-MS[16]. Although HPLC methods and enzymatic are generally reliable enough some differences in laboratory efficiency still exist[17]. In general, these methods present little flexibility and portability and they are time consuming.

Electrochemical sensors are ideal systems to monitor compounds of biological interest using portable devices and have been previously proved successful for these tasks. In the same way glucose sensors are now used in routine analysis, in both clinical or home glucose control, a future portable system able to monitor the concentration of B_6 vitamers in biological fluids could be of great help to maintain adequate levels of these compounds through diet.

The vast majority of the electrochemical studies found in the literature have been performed in mercury electrodes and have been focused in understanding the electrochemistry of these compounds and how the different organic functions associated to them behave under different conditions[18-20]. How these B6 vitamers behave under physiological conditions (pH 7) have been studied by Gonzalez-Rodriguez et al[21] and results on their electrochemical behaviour were obtained. This study offered information on a variety of electrochemical parameters such as, for example, number of electrons exchanged, coefficient of diffusion for the different compounds and other valuable electrochemical data to infer reversible or irreversible behaviour. Mercury electrodes proved to be an exceptional tool to gather electrochemical information but cannot be used in the design of portable sensors. Only one voltammetric study on vitamin B6 can be found on the analysis of pharmaceutical compounds and standards[22]. This study only focused on the study of pyridoxine (pH 6), which is the form in which vitamin B-6 is presented in tablets and could not identify any of the biological forms of vitamin B-6 (Pyridoxal-5'-phosphate, Pyridoxamine or Pyridoxic Acid) or reaction mechanisms for these substances in the electrodes, showing no potential use in biological fluids or conditions similar to biological conditions and therefore not useful in sensor development.

The work developed here presents a comparative study on the reduction of these compounds on glassy carbon electrodes. Reaction mechanisms are suggested and the irreversible nature of these processes is also studied. Electrochemical data on reduction of these compounds have been also obtained for their potential use in the design and build of sensors and modified electrodes for use in portable systems. The study of the family of compounds in solid interfaces could also be of great use and provide with very useful information to understand how these substances are operating in biological environments and the mechanism involved in their electrochemical reactions.

2. MATERIALS AND METHODS

2.1. Apparatus and instruments

A computer assisted Inelecsa PDC1212 potentiotast was used for cyclic voltammetry (CV) (Inelecsa, Seville, Spain). For additional voltammetry studies, an AMEL electrochemistry instrument model 433-A fitted with homemade software and a Computrace 957 (Metrohm, Herisau, Switzerland) were also used. The experiments were conducted in a double-walled Metrohm E-505 thermostatic cell fitted with a three electrode system: a saturated calomel reference electrode (SCE) Ingold 303-NY or alternatively with a Potassium Chloride reference electrode, a platinum auxiliary electrode and, as working electrode, a 3mm diameter glassy carbon electrode all from Metrohm.

The concentration of some compounds, such as PLO and PLPO, were monitored by UV-Vis spectrophotometry using a Perkin Elmer Lambda 3B spectrophotometer (Perkin Elmer, Massachusetts, USA).

Temperature for both electrochemical and spectrophotometric experiments was kept constant at 25±0.1ºC by using an thermostatic bath Selecta Frigiterm, model S-382 (Selecta, Barcelona, Spain). The pH was monitored using a Crison 2001 pH-meter (Crison, Barcelona, Spain).

2.2 Reagents

Pyridoxine (PN), 4-pyridine aldehyde (P), pyridoxal (PL), pyridoxal-5'-phosphate (PLP), 4-pyridine aldehyde oxime (PO), 4-pyridin carboxylic acid (PC), 4-pyridoxic acid (PA), amino methyl pyridine (AP), pyridoxamine (PM), pyridoxamine-5'-phosphate (PMP) and $K_3Fe(CN)_6$ were purchased from Sigma (Schnelldorf, Germany)

4-pyridoxic acid lactone (PAL) was synthesised by acidic hydrolysis of PA. A sample of PA in 1 mM HCl was incubated at 37ºC and after 24h the conversion into lactone was complete. The reaction was followed by monitoring absorption spectra at pH 6, the initial band centred at 315 nm changes to 356 nm as the lactone is formed[23].

Pyridoxal oxime (PLO) and pyridoxal-5'-phosphate (PLPO) were synthesised using a method used by Pocker et al[24].

Other reagents purchased from Merck (New Jersey, USA) were phosphoric acid, acetic acid, hydrochloric acid, sodium hydroxide and potassium chloride. Acetic/acetate and hydrogen phosphate/di-hydrogen phosphate buffers were used for the experiments where pH needed to be kept constant.

2.3. Measurements

Prior to analysis, Oxygen from the solution was eliminated by bubbling nitrogen for 10 minutes. Temperature was kept at 25C for all the experiments. The glassy carbon electrode was cleaned with a paste made of de-ionised water and 0.3 μm alumina for a minute in between analyses. The electrode was rinsed with water and placed in an ultrasound bath for 3 minutes.

The area of the graphite electrode was calculated experimentally by using a $K_3Fe(CN)_6$ 1mM solution in KCl 1 M. The equations used for the determination of the experimental area were:

$$i_p=(2.69x10^5)\ n^{3/2}AD_o^{1/2}v^{1/2}c_o \qquad (1)$$

$$i_p=(2.99x10^5)\ n(\alpha n_a)^{1/2}AD_o^{1/2}v^{1/2}c_o \qquad (2)$$

being (1) the typical equation for a reversible process and (2) that used for an irreversible process and where i_p= peak intensity, n=number of electrons exchanged, A=area, D_o= coefficient of diffusion, v=scan rate, α=coefficient of the electronic transfer and C_o=concentration.

As the value for the coefficient of diffusion for $K_3Fe(CN)_6$ is $D_o=7.84x10^{-6}cm^2/s$, the calculated area values for (1) and (2) were 0.0691 y 0.0711 cm^2, respectively, which are in good agreement with a geometrical area of 0.0706 cm^2. All the intensity of current values given, normalised to consider the surface of the electrode, have been calculated using the geometric area.

3. RESULTS AND DISCUSSION

3.1. Reduction on glassy carbon electrodes

A summary of the results obtained in the study of the reduction of the different organic functions present for the compounds of the family of the vitamin B6 using a glassy carbon and cyclic voltammetry is presented in Table 1.

Pyridoxine shows a reduction peak close to the support electrolyte discharge with a peak potential close to -1.7 V with a limiting current according to a bielectronic reduction process. This suggests that it could correspond to the reduction of the hydroxyl group present in the molecule to saturated analogue. It is interesting to note that this peak is not observed in mercury at pH 7 by the presence of the catalytic wave close to the discharge of the support electrolyte[21].

The aldehyde functions present in the molecules of PL, PLP and P yield a reduction peak around -0.85 V. In the case of P a second electrodic process is observed at -1.7 V, which corresponds to the reduction of the alcohol formed as a reduction product of the reduction of the aldehyde, which also matches the potential obtained for the reduction of hydroxyl group in pyridoxine. The two reduction peaks observed for the mercury electrode[21] fuse in a single one when using a glassy carbon electrode. In all cases the three molecules present a lower limiting current to the corresponding for the bielectronic reduction of the aldehyde group (the apparent number of electron, n, is between 0.5 and 0.8). This fact can be explained by the hydration of aldehyde group[25]. On mercury electrode a catalysis intramolecular in the dehydration reaction yield the bielectronic wave whereas on hydrophobic graphite surface the reaction appears as not catalysed.

The reduction of the oximes yield two peaks (Figure 1), as in the case for mercury electrodes[21], the first corresponding to the tetra-electronic reduction to the correspondent amine (in

the range -1.0 to -1.2 V) and the second for the bielectronic reduction of the amine (in the range -1.6 to -1.65 V).

Table 1. Results for Cyclic Voltammetry for c=1mM at pH=7

Compound	$-E_p$/mV	J_p/μA/cm^2	n	E_p-$E_{p/2}$/mV	α_{na}
PN (KCl0.1M)	1698	314	1.8	82	0.6
P (KCl0.1M)	825/1698	123/149	0.5/0.6	62/84	0.8/0.6
PL (KCl0.1M)	882	108	0.5	67	0.7
(KCl 1M)	882	120	0.6	63	0.8
(TEAP0.1M)	900	117	0.5	83	0.6
PLP (KCl0.1M)	877	123	0.7	62	0.8
(KCl 1M)	861	141	0.8	62	0.8
(TEAP0.1M)	848	123	0.7	69	0.7
PO (KCl0.1M)	1120/1580	791/383	4/1.6	104/72	0.46/0.66
(KCl 1M)	1120/1592	713/371	3.6/1.6	112/72	0.43/0.66
(TEAP0.1M)	1120/1560	805/345	4.1/1.5	96/64	0.50/0.74
PLO (KCl0.1M)	1074/1624	754/400	3.5/1.9	67/79	0.71/0.60
(KCl 1M)	1033/1616	710/400	3.3/1.9	67/73	0.71/0.65
(TEAP0.1M)	1074/1632	741/358	3.4/1.7	64/64	0.71/0.74
PLPO (KCl0.1M)	1132/1666	647/323	3.5/1.9	102/92	0.47/0.52
(KCl 1M)	1128/1664	631/323	3.4/1.9	103/90	0.46/0.53
PA (KCl0.1M)	1328	601	3.0	43	1.1
(KCl 1M)	1335	566	2.8	43	1.1
(TEAP0.1M)	1335	495	2.5	48	1.0
PAL (KCl0.1M)	1022	594	2.9	45	1.1
AP (KCl0.1M)	1585	456	2.1	85	0.6
PM (KCl0.1M)	1570	410	1.8	67	0.7
(KCl 1M)	1590	466	2.1	69	0.7
(TEAP0.1M)	1608	447	2.0	59	0.8
PMP (KCl0.1M)	1576	1004	1.8	94	0.5
(KCl 1M)	1569	1132	2.0	93	0.5

E_p, peak potential at 0.1 V/s
J_P , current density
n: electron number calculated by irreversible process model
E_p-$E_{p/2}$; peak potential half peak potential difference.
$\alpha_{na} = 47.6/(E_p-E_{p/2})$

The reduction of the carboxylic group observed for PA and its lactone (PAL) is clearly defined in the potential range close to -1.3 V for the former (Figure 1) and -1 V for the latter. The limiting current is also lower than expected for the tetralectronic reduction observed in mercury electrode due to the reaction of hemiacetal ring cleavage to PL hydrate intermediate followed by a slow dehydration reaction to obtain the free aldehyde[23].

Amines also show a bielectronic reduction peak around -1.5V with the limiting current diffusion controlled on the top of the wave.

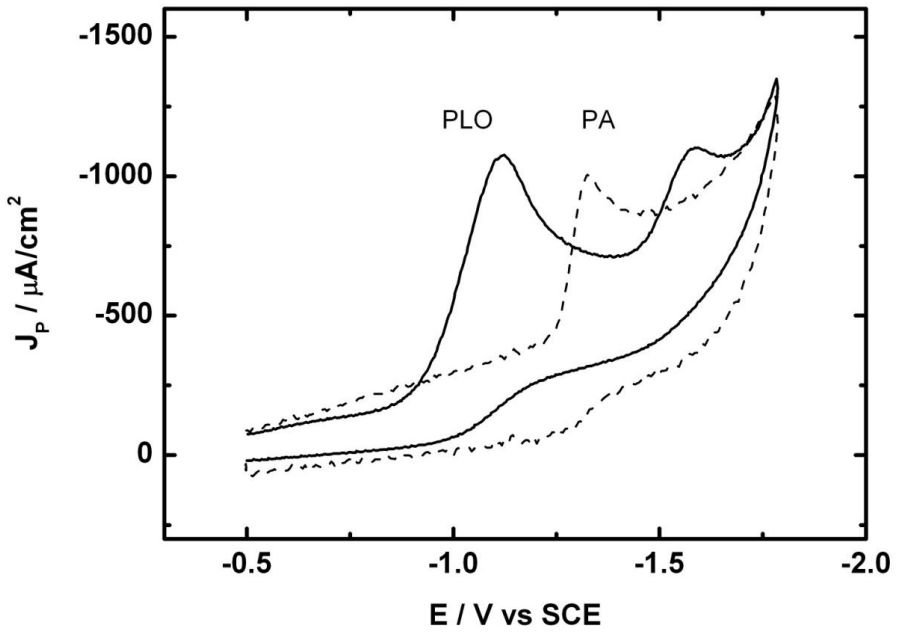

Figure 1. Cyclic Voltammetry. Pyridoxal oxime (——) and pyridoxic acid (- - -). c=1mM and V= 01.V/s

An interesting observation is the fact that the B6 compounds studied in this work show irreversible electrochemical reduction behaviour. In all cases the first electron transfer ($\alpha_{na} < 1$) is the responsible of the kinetic control of the process, with the exception of the PA and its lactone (PAL) where the second electron transfer is the controlling step ($\alpha_{na} \geq 1$). This fact is different from the observed one on mercury electrode, where for the majority of the vitamin B6 derivatives, the second electron transfer is the controlling step[21].

On the other hand, the concentration and the nature of the supporting electrolyte does not produce an appreciable effect on current intensity and peak potential in all cases.

The analytical characteristics of the most important members of vitamin B6 family are summarised in table 2. These compounds represent the most interesting members of the family from a biological point of view and those for which development of a sensor for their detection in biological fluids would prove useful. All the compounds presented good behaviour for the different analytical properties for each of the analysed methods. A factor influencing the potential use of this electrode as B-6 vitamers sensor is the presence of a matrix effect observed when PLP, PL, PA were subjected to analysis in urine samples (this effect was not observed in aqueous solutions). Blank samples were run prior to analysis to establish the baseline and to be able to establish positive responses to the presence of these analytes. The methodology followed to minimise potential errors associated to this matrix effect used of the standard addition method where the samples where spiked with known concentrations of the analyte of interest, depending on their LoD and linear range. Interestingly, this matrix effect was not observed for PM and the values obtained for the standard addition method were

comparable to values obtained using the calibration curve and direct analysis of the urine samples. For the rest of the analytes described, the use of the standard addition method proved successful and good precision and recoveries were obtained when analysing urine samples

Table 2. Analytical parameters for the analysis of B-6 vitamers using glassy carbon electrodes. Recoveries are referred to urine analysis using standard addition.

Compound	Calibration equations	LOD (µM)	RSD (%) (n=5)	Recovery (%)	Range (µM):
PLP	nA = 11.92[µM] + 18.14 R^2 = 0.999	59	4.11	97.7 (400 µM)	100-800
PA	nA = 23.90[µM] - 756.6 R^2 = 0.989	19	4.98	98.9 (150 µM)	40-200
PM	nA = 0.71[µM] + 685.4 R^2 = 0.990	286	6.45	104.4 (1000 µM)	500-1600
PL	nA = 0.45[µM] + 7.699 R^2 = 0.998	99	0.45	99.7 (450 µM)	200-1000

Figure 2 shows the voltammograms obtained at several concentrations for the PA, PL, PLP and PM using diferential pulse voltammetry.

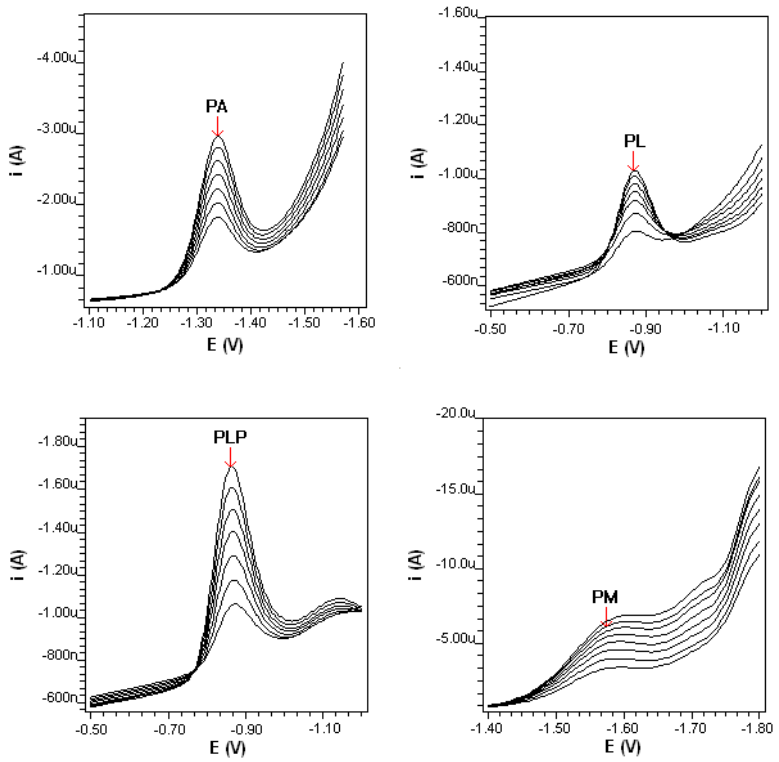

Figure 2. Cocentration study of the main B-6 vitamers using Diferential Pulse Voltammetry: (a) PA (b) PL (c) PLP and (d) PM

The results for these analyses show that there is potential to use glassy carbon electrodes for the analysis of these B-6 vitamers both in aqueous solution and in biological fluids, even if the matrix has an effect on the response of the electrode. The reduction process for each analyte is specific enough to assign the peaks to the reduction of the compound under study with a biological meaning. Only in the case of PL and PLP the two reduction peaks are close enough to interfere if both are present. Even in this was the case, a joint concentration value for both substances would still be valuable as both substances are involved in a phosphorylation equilibria involving a kinase and ATP in which Pyridoxal is readily transformed into Pyridoxal-5'-Phosphate. The latter is the active form of vitamin B6 in the body and the one presenting real biological interest. Figure 2 and Table 2 show the good linearity achieved by all these compounds and how an increase in concentration is followed by a linear increase of the response in the range analysed.

4. CONCLUSIONS

A novel electrochemical method for the analysis of B-6 vitamers has been presented. The methodology proposed in this work based on the use of glassy carbon electrodes to monitor vitamin B-6 compounds with biological interest has proved to be efficient in their detection and quantitation. A close view on the reaction mechanism of these compounds on glassy carbon concluded that most of the B6 compounds studied in this work show irreversible electrochemical reduction behaviour. In almost all cases (with exception of PA and PAL with $\alpha_{na} \geq 1$) the responsible of the kinetic control of the process is the first electron transfer ($\alpha_{na} < 1$).

The analytical results for the limits of detection, linearity ranges, precision and recovery percentages proved that the method can be successfully used to determine the most important component in both model solutions and biological fluids.

References

1. S.C. Larson, N. Orsini, A. Wolk, *JAMA*, 303(11) (2010) 1077
2. L. Chih-Cheng, *JAMA*, 303(22) (2010) 2251
3. S.C. Larson, N. Orsini, A. Wolk, *JAMA*, 303(22) (2010) 2252
4. R.Z. Stolzenberg-Solomon, D. Albanes, F.J. Nieto, T.J. Hartman, J.A. Tangrea, M. Rautalahti, J. Sehlub, J. Virtamo , P.R. Taylor, *J. Natl. Cancer Inst*, 91(6) (2010) 535
5. T.J. Hartman, K. Woodson, R. Stolzenberg-Solomon, J. Virtamo, J. Selhub, M.J. Barrett, D. Albanes,
6. *Am. J. Epideomi*, 153 (2001) 688
7. Z.F. Zhang, R.C. Kurtz, G.P. Yu, M. Sun, N. Gargon,M. Karpeh Jr, J.S. Fein, S. Harlap, *Nutr. Cancer*, 27 (1997) 298
8. E. Negri, S. Franceschi, C. Bosetti, F. Levi, E. Conti, M. Parpinel, C. La Vecchia, *Int. J. Cancer*, 86 (2000) 122
9. S. Friso, D. Girelli, N. Martinelli, O. Olivieri, V. Lotto, C. Bozzini, F. Pizzolo, G. Faccini, F. Beltrame, R. Corrocher, *Am. J. Clin. Nutr.*, 79 (2004) 992
10. Y.Schwammenthal, D. Tanne, *Lancet Neurol* ., 3 (2004) 493

11. M.E. Rybak, C.M. Pfeiffer, *Anal Biochem,* 333 (2004) 336
12. I.Baranowska, J. Płonka, *J.Liq.Chrom.Rel.Tech,* 31 (19) (2008) 2974
13. M.L. Marszałł, A. Lebiedzińska, W. Czarnowski, R. Makarowski, M. Kłos, P. Szefer, *J. Chrom. B,* 877 (27) (2009) 3151
14. A.T. Vasilaki, D.C. McMillan, J. Kinsella, A. Duncan, D. St J O'Reilly, D. Talwar, *Am. J. Clin. Nutr.*, 88 (2008) 140
15. M.R. Bisp, M. Vakur Bor, E.M. Heinsvig, M.A. Kall, E. Nexø, *Anal. Biochem,* 305 (1) (2002) 82
16. Q. Han, M. Xu, L. Tang, X. Tan, X. Tan, Y. Tan, R.M. Hoffman, *Clin Chem,* 48 (2002) 1560
17. P.M. Ueland, Ã. Midttun, A. Windelberg, A. Svardal, R. SkÃ¥levik, S. Hustad, *Clin. Chem. Lab. Med*., 45 (12) (2007) 1737
18. M.E.Rybak, R.B. Jain, C.M. Pfeiffer., *Clin. Chem.*, 51 (7) (2005) 1223
19. M. Blázquez, L. Camacho, M. Jiménez, M. Domínguez, *J.Electroanal. Chem.,* 189 (1985) 195
20. R. izquierdo, M. Blázquez, M. Domínguez, F. García-Blanco, *Bioelectrochem. Bioenerg.*, 12 (1984) 25
21. A.J. Román, J.M. Sevilla, T. Pineda, M. Blázquez, *J. Electroanal. Chem.*, 410 (1996) 15
22. J. Gonzalez-Rodriguez, J.M. Sevilla, T. Pineda, M. Blázquez, *Russ. J.Electrochem.,* 47 (2011) 835
23. R.C. Barthus, L.H. Mazo, R.J.Poppi., *J. Pharm. Biomed. Anal.*, 38(1) (2005) 94
24. T. Pineda, M.I. López-Cozar, J.M. Sevilla, M. Blázquez, *J.Electroanal. Chem.*, 403 (1996) 101
25. A.Pocker, E.H. Fisher, *Biochemistry,* 8 (1969) 5181
26. T. Pineda, M. Blázquez, R. Izquierdo, M. Domínguez, F. García-Blanco, *J.Chim.Phys.*, 88 (1991) 371