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Short communication

Enzyme immunoassay technique using alkaline phosphatase enzyme labels and a Nafion electrode as sensor[1]

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1. Introduction

Alkaline phosphatase (AP) is widely used as an enzyme label in enzyme-linked immunosorbent assay (ELISA) and it has been used by several groups to develop electrochemical enzyme immunoassay at a bare electrode (see, e.g., [1–8]). In order to increase sensitivity even further, flow injection analysis [1,3,4] and enzymatic recycling [6,7] were developed.

The sensitive electrochemical detection of AP [8] and AP labels [9] has recently been improved in our group by using a Nafion-modified glassy carbon electrode (GCE) in connection with an anionic substrate S^- and a procationic electroactive product P^+ of the enzyme reaction, as sketched in Fig. 1 for an ELISA. The polyanionic perfluorosulfonated Nafion polymer is known for its ability to accumulate cationic or procationic species and concomitantly expel anionic compounds. Therefore, the product P^+ of the enzyme reaction was accumulated, whereas the initial substrate S^- could not penetrate into the film. The square-wave voltammetric (SWV) detection of AP could be achieved with a detection limit of 0.02 U l^{-1} [8] (2.8×10^{-14} M of bovine intestinal mucosa AP). Phenytoin at therapeutic concentrations in 1 μl of clinical sample could thus be determined using the ELISA procedure, a Nafion-modified GCE and the **1–2** substrate–product couple shown in Table 1 [9].

In order to optimize the experimental conditions, a Nafion-incorporated carbon paste electrode was built and its permselectivity was examined, Moreover, the **3–4**, **5–6** and **7–8** couples shown in Table 1 were prepared and their accumulation was compared. Finally, AP assays were carried out.

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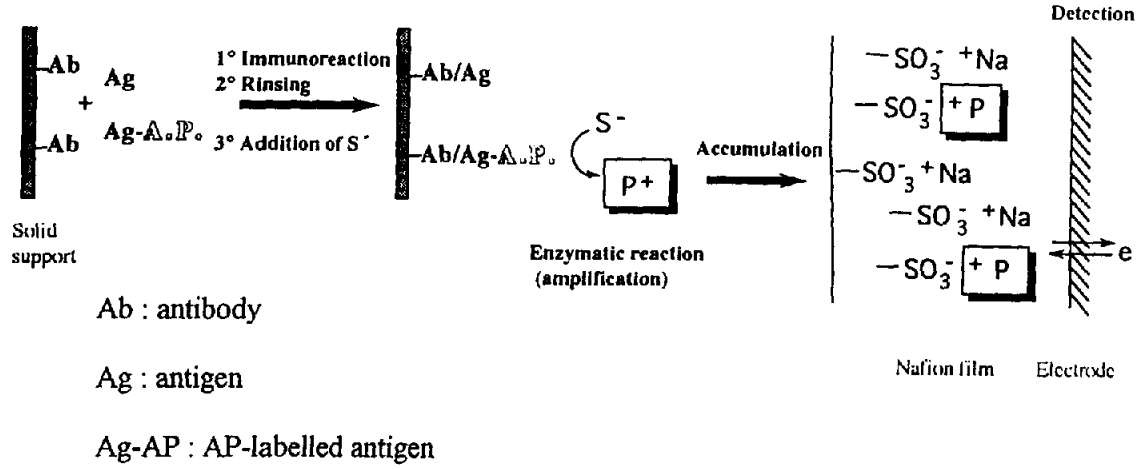


Fig. 1. Schematic representation of an ELISA using an AP enzyme label and a Nafion-modified GCE.

2. Experimental

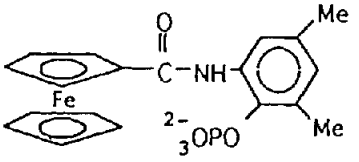
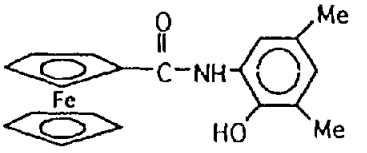
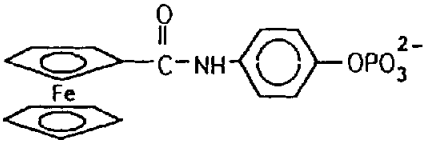
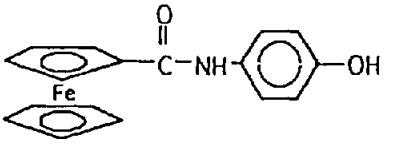
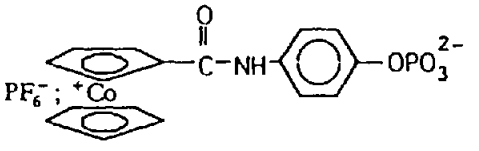
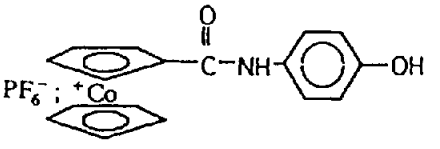
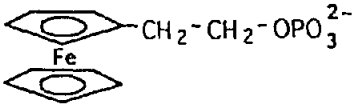
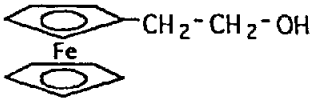
A 5% (w/w) Nafion solution (EW1100) was purchased from Aldrich, AP from bovine intestinal mucosa (ref. P5521) was provided by Sigma and (ferrocenylmethyl)trimethylammonium iodide was supplied by Lancaster. The synthesis of **1** and **2** was described previously [8] and compounds **3** and **4** were prepared using the method reported by McNeil et al. [2]. The synthesis of **5–8** will be described elsewhere [10]. Two buffer solutions were used, i.e. a phosphate buffer (PB) (pH 7.4; NaH_2PO_4 8.7×10^{-3} M, Na_2HPO_4 30.4×10^{-3} M, NaCl 5×10^{-2} M) and a Tris buffer (TB) (pH 9.0, Tris 5×10^{-2} M, $MgCl_2$ 1×10^{-2} M, NaCl 5×10^{-2} M). The Nafion-modified GCE preparation, the electrochemical instrumentation and the experimental conditions for SWV were described previously [11]. All the potentials were referred to a Ag/AgCl(Cl^-, 0.05 M) reference electrode. For the accumulation and detection procedures, the rotating electrode was exposed to the non-deaerated solution and immediately rotated at 600 rpm for 5 min, then a potential scan was applied and the resulting peak current obtained by SWV or linear-scan voltammetry (LSV) was taken as the analytical response. For the incubation procedure, substrate **5** or **7** was incubated in TB solution (1 ml) with AP in a capped glass maintained at 25°C in a thermostated bath (for **7**) or at room temperature (for **5**).

3. Results

Previous AP enzyme immunoassays of phenytoin were performed with a Nafion-modified GCE as sensor and **1** as the enzyme substrate. The three-step procedure required successive pH changes. In step I, the competitive immunoreaction was performed in physiological neutral medium. In step II, the enzyme generation of **2** took place after addition of **1** in alkaline buffered solution (pH 10.2) containing an Mg^{2+} salt. In step III, the accumulation of **2** into the modified electrode proceeded by applying a potential of 0.6 V vs. Ag/AgCl for 5 min in neutral medium (pH 7.5) and was followed by SWV scan. Substrate **1** was dianionic and therefore was repelled from the anionic Nafion film, whereas **2** was entrapped within the Nafion film as a ferrocenium salt, and so the sensitive electrochemical detection of **2** was possible (detection limit, 10^{-8} M). Despite its advantages, this method suffered from a drawback related to the design of a single-use electrode, since the hydrophobic ferrocene-labeled alcohol **2** irreversibly accumulated within the Nafion film, and so the electrode could not be reused.

In a search for a single-use sensor of low cost, modified carbon paste electrodes (CPE) are attractive, since a fresh modified electrode surface can be generated in a rapid and quantitatively

Table 1
Substrate–product couples

S^-	P^+
 1	 2
 3	 4
 5	 6
 7	 8

reproducible fashion [12]. A Nafion-incorporated CPE was contructed and optimization of its SWV response at the virgin surface was examined with (ferrocenylmethyl)trimethylammonium iodide (9)

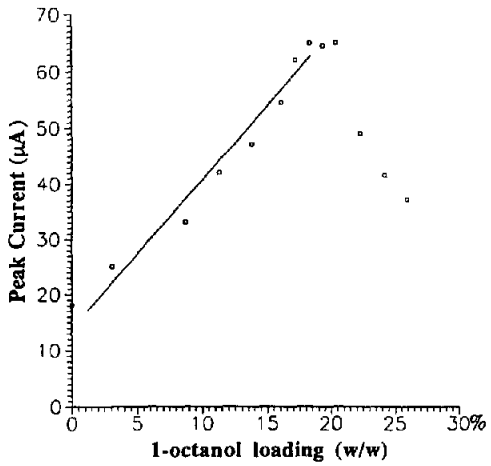


Fig. 2. Effect of 1-octanol in CPE with 8.8% neutralized Nafion loading on the SWV peak current of **9** (1 μM in PB).

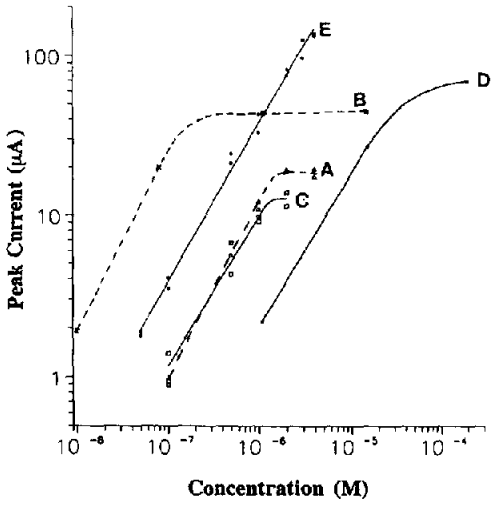


Fig. 3. Variation of the SWV peak current intensity with the concentration of **2** (curves A and B), **4** (curves C and D) and **6** (curve E) at a Nafion-modified CPE (curves A, C and E) and Nafion-coated GCE (curves B and D).

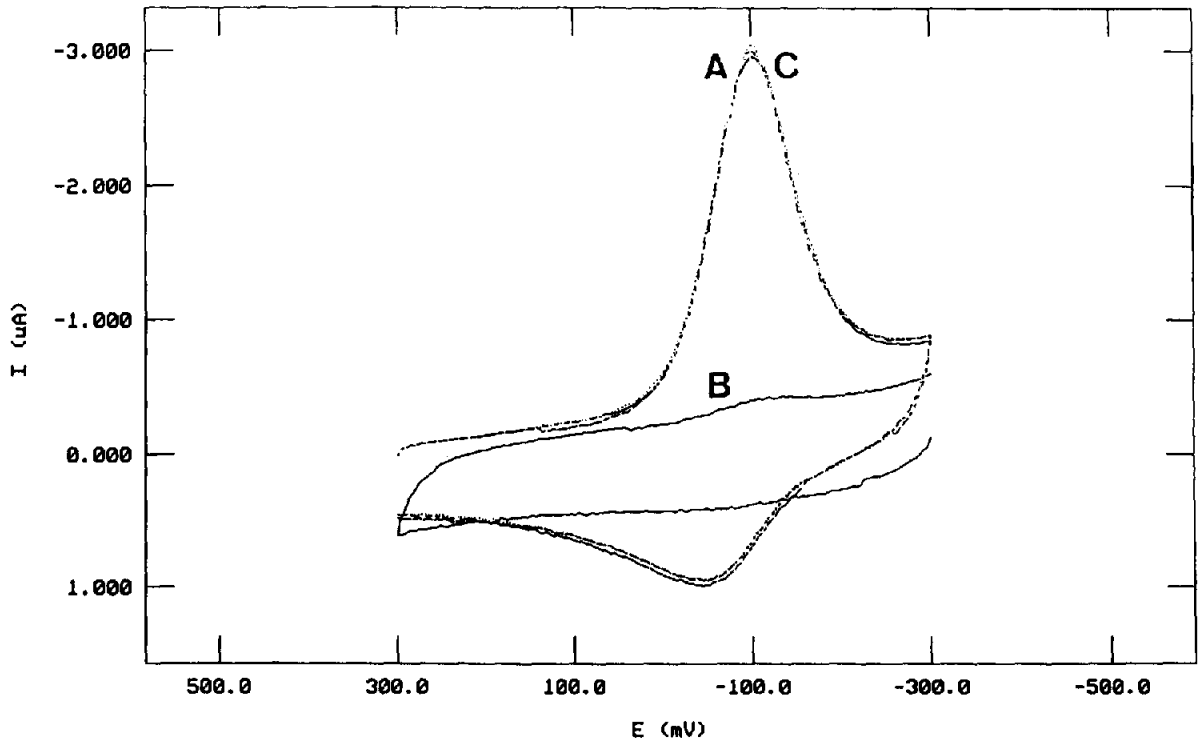


Fig. 4. LSV curves (scan rate 100 mV s^{-1}) obtained in TB for **8** (2 μM) at a Nafion-modified GCE after accumulation at 0.3 V (curve A), followed by electrochemical cleaning at −0.3 V for 2 min (curve B) and a second accumulation at 0.3 V (curve C).

as a model of an irreversibly accumulated cation. Reproducible and sensitive responses could be obtained without preconditioning by incorporation of 1-octanol and neutralized Nafion into the paste. Addition of the former increased the current response (Fig. 2) and of the latter gave lower background currents and more stable modified electrodes than with non-neutralized Nafion. The carbon paste composition was progressively modified to improve the SWV response of **9** (1 μM) and the following optimized composition (w/w) was finally adopted: 39.4% graphite powder, 20.9% 1-octanol, 32.8% silicone oil and 6.9% neutralized Nafion. The peak intensity (ca. 100 μA) was 470 times higher than in the absence of Nafion. After each measurement, the CPE surface was renewed by pushing a small amount of the paste out of the electrode body and the new surface obtained after cutting was polished on a Teflon sheet. A statistical analysis showed that the relative standard deviation between each new electrode surface was ca. 5%.

Prior to this work, a CPE containing non-neutralized Nafion and a complexing reagent as modifiers was employed for the determination of trace amounts of metal ions [13].

3.1. Comparative accumulation of the alcoholic products at Nafion-modified CPE and GCE

Fig. 3A shows the calibration curve obtained by SWV for **2** (PB pH 7.4, applied potential 0.6 V) at the optimized Nafion-modified CPE. As expected, the SWV peak current, i_p, increased with increasing concentration to reach a limiting value above 2×10^{-6} M, suggesting the saturation of the available sulfonated sites of the Nafion by the ferrocenium moieties. The detection limit (signal-to-noise ratio = 3) was 1×10^{-7} M,

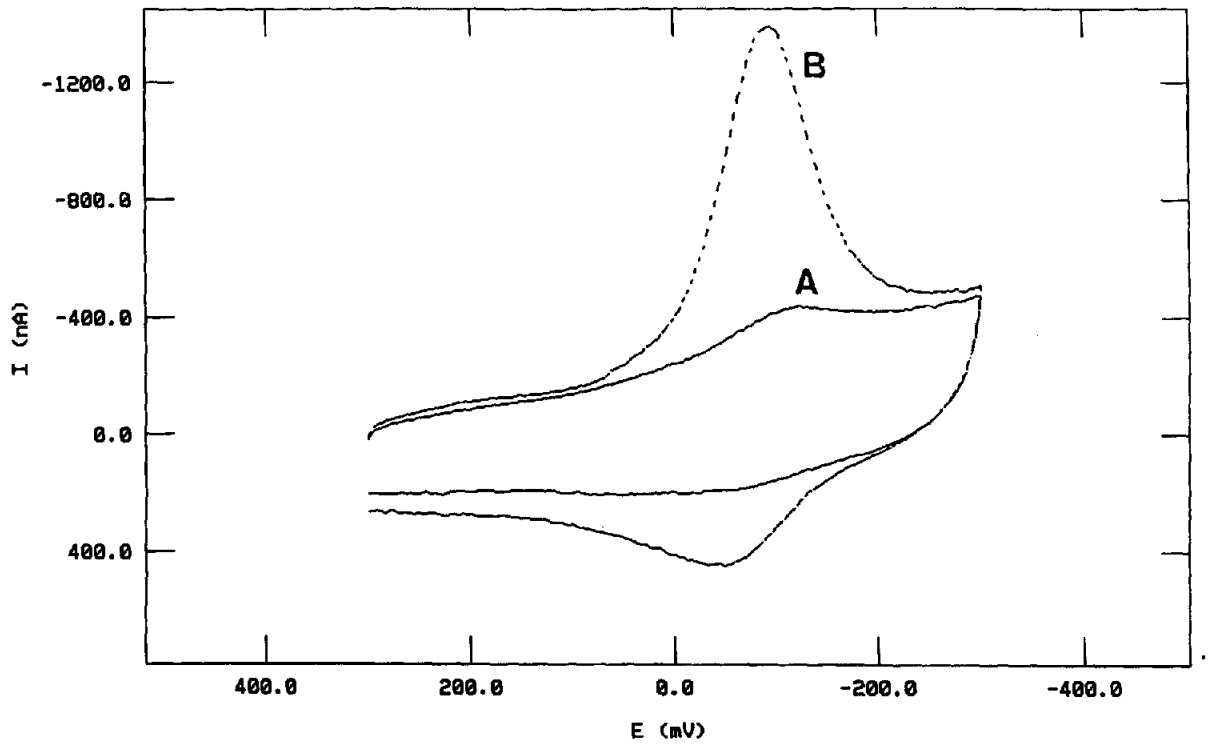


Fig. 5. LSV curves (scan rate 100 mV s^{-1}) obtained in TB for **7** (10 μM) (curve A) and **8** (1 μM) (curve B) at a Nafion-modified GCE. Accumulation at 0.3 V.

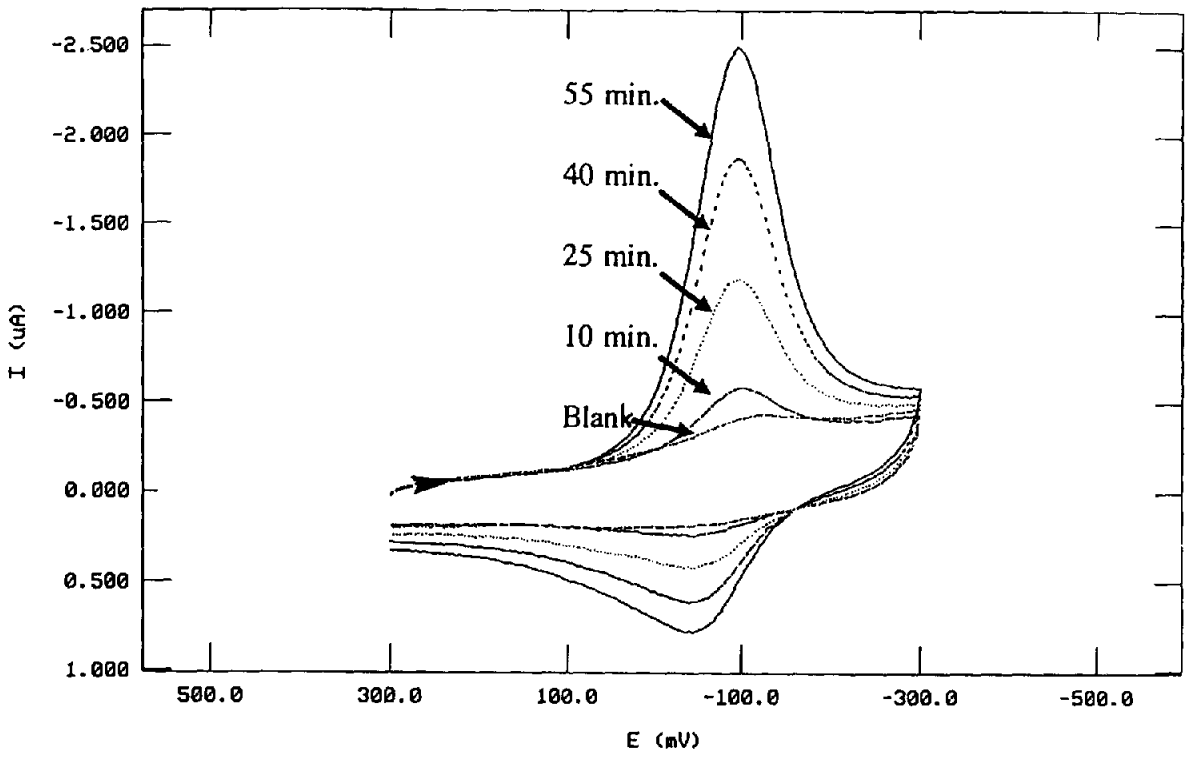


Fig. 6. LSV curves (scan rate 100 mV s^{-1}) obtained at a Nafion-modified GCE in TB + 1 mM $MgCl_2$, after incubation of 7(1×10^{-6} μM) and AP (8×10^{-11} M) for several periods. Electrochemical cleaning at −0.3 V for 2 min was carried out between measuring each curve.

whereas it was 1×10^{-8} M at a Nafion-modified GCE under the same experimental conditions (compare curves A and B). Conversely, the calibration curve for **4** was more sensitive at a Nafion-modified CPE than GCE (compare curves C and D). The cationic alcohol **6** accumulated in open circuit at a Nafion-modified CPE and led to the calibration curve E. The cathodic reduction of the oxygen traces contained in the non-deaerated PB solution interfered with the reversible one-electron reduction of the cobaltocenium function contained in **6** (standard potential −1.1 V). Therefore, the SWV calibration curve of **6** was plotted by scanning the potentials from −1.3 to −0.6 V after accumulation. Fig. 3 clearly shows that the most sensitive detection limit at a Nafion-modified CPE was obtained with this salt (5×10^{-8} M).

The LSV curves in Fig. 4 were obtained at a Nafion-modified GCE. They show that the accumulation of the hydrophilic alcohol **8** proceeded reversibly, since it was possible to expel it from a Nafion film by rotating the electrode and applying a negative potential (−0.3 V) for 2 min after accumulation of the ferrocenium salt. Therefore, the Nafion-modified GCE could be renewed after accumulation of **8**, whose detection limit reached 5×10^{-8} M by LSV. Moreover, the LSV method is preferable to the SWV method for this alcohol.

3.2. Nafion selectivity

The specificity of the technique was based on the Nafion selectivity and therefore on the preferential preconcentration of P^+ compared with S^-. This is demonstrated in Fig. 5 which shows the LSV curves obtained at a Nafion-modified GCE

immersed in a solution of **7** ($C = 1 \times 10^{-5}$ M) or **8** ($C = 1 \times 10^{-6}$ M) of pH 7.9 (Tris buffer). The peak intensity for **7** was lower than that for **8**, although **7** was more concentrated. In other words, the ratio i_p/C was 90 times higher for **8** than for **7**, which indicates good selectivity of the Nafion film. Even better results were obtained with the **5–6** couple at a Nafion-modified CPE under the same experimental conditions: the ratio i_p/C was 230 times higher for **6** than for **5**.

3.3. AP assays

The LSV curves in Fig. 6 were obtained at a reusable Nafion-modified GCE after incubation of **7** (1.0×10^{-5} M) and AP (60 U l^{-1}, 7.8×10^{-11} M) in a TB solution containing Mg^{2+}. The peak current increased linearly with the incubation time (slope $p = 4 \times 10^{-8}$ A min^{-1}). The calibration curve for AP (p versus AP concentration) indicated a detection limit of 1.5 U l^{-1} (2×10^{-12} M). The value is lower at a Nafion-modified CPE (0.5 U l^{-1}, 7×10^{-13} M) and preliminary results with **5** indicate an even lower value.

4. Conclusion

The most suitable couples for AP assays at Nafion-modified CPE and GCE are **5–6** and **7–8** respectively. More sensitive results were obtained previously at the latter electrode using the **1–2** couple. However, this electrode could not be reused.

References

[1] R.Q. Thompson, M. Porter, C. Stuver, H.B. Halsall, W.R. Heineman, E. Buckley and M.R. Smith, Anal. Chim. Acta, 271 (1993) 223–229, and references cited therein.
[2] C.J. McNeil, I.J. Higgins and J.V. Bannister, Biosensors, 3 (1987–88) 199–209.
[3] J.E. Frew, N.C. Foulds, J.M. Wilshere, N.J. Forrow and M.J. Green, J. Electroanal. Chem., 266 (1989) 309–316.
[4] Z. Yu, Y. Xu and M.P.C. Ip, J. Pharm. Biomed. Anal., 12 (1994) 787–793.
[5] P.H. Treloar, A.T. Nkohkwo, J.W. Kane, D. Barber and P.M. Vadgama, Electroanalysis, 6 (1994) 561–566.
[6] S. Yamaguchi, S. Ozawa, T. Ikeda and M. Senda, Anal. Sci., 8 (1992) 87–88.
[7] L. Della Ciana, G. Bernacca, F. Bordin, S. Fenu and F. Garetto, J. Electroanal. Chem., 382 (1995) 129–135.
[8] A. Le Gal La Salle, B. Limoges and C. Degrand, J. Electroanal. Chem., 379 (1994) 281–291.
[9] A. Le Gal La Salle, B. Limoges and C. Degrand, Anal. Chem., 34 (1995) 1245–1253.
[10] S. Rapicault, B. Limoges and C. Degrand, to be published.
[11] B. Limoges, C. Degrand, P. Brossier and R.L. Blankespoor, Anal. Chem., 65 (1993) 1054–1060.
[12] K. Kalcher, Electroanalysis, 2 (1990) 419–433.
[13] Z. Gao, P. Li, G. Wang and Z. Zhao, Anal. Chim. Acta., 241 (1990) 137–146.