Amperometric separation-free immunosensor for real-time environmental monitoring

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Abstract

Immunoanalytical techniques have found widespread use due to the characteristics of specificity and wide applicability for many analytes, from large polymer antigens, to simple haptens, and even single atoms. Electrochemical sensors offer benefits of technical simplicity, speed and convenience via direct transduction to electronic equipment. Together, these two systems offer the possibility of a convenient, ubiquitous assay technique with high selectivity. However, they are still not widely used, mainly due to the complexity of the associated immunoassay methodologies. A separation-free immunoanalytical technique is described here, which has allowed for the analysis of atrazine in real time and in both quasi-equilibrium and stirred batch configurations. It illustrated that determinations as low as 0.13 μM (28 ppb) could be made using equilibrium incubation with an analytical range of 0.1–10 μM. Measurements could be made between 1 and 10 mM within several minutes using a real-time, stirred batch method. This system offers the potential for fast, simple, cost-effective biosensors for the analysis of many substances of environmental, biomedical and pharmaceutical concern.

Keywords: Immunosensor; Atrazine; Polyaniline; Real-time

1. Introduction

The immunoassay format remains a robust, ubiquitous analytical tool, finding application in a broad range of areas such as the environment and biomedicine. It is applicable to a wide range of analytes from whole cells [1], large polymers [2], haptens [3] and even single atoms [4] because of the available antibody production methodologies [5]. Immunoassays are highly sensitive, selective, resistant to interference and relatively cost-effective. The two main methodologies for immunoassay design are homogeneous and heterogeneous [6]. In elegance, homogeneous assays are superior, as they can be performed in solution in a single step. They are, however, relatively insensitive, and cannot always achieve the desired measurement limits. Thus, the assay of choice in most instances has been the heterogeneous immunoassay format, e.g. ELISA. Heterogeneous systems can be performed in a variety of ways. For large molecules such as proteins,

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the most common is the sandwich assay. For small, hapten molecules (<10 kDa), the method of choice is the competition immunoassay. Both methodologies require a series of reagent additions, separation and washing steps to differentiate between materials that are taking place in the immunological reaction and those that are not. In addition, most assays are performed under equilibrium or quasi-equilibrium conditions. This means that immunoreagents must be incubated together for periods. This combination of multiple steps and incubation times means that immunoassays are relatively slow (taking hours) and require a complex series of manipulations, requiring either skilled technicians or complex instrumentation.

Sensors and biosensors in particular are revolutionising modern analysis mostly because of the two characteristics of speed and simplicity. The application of a sample to a biosensor brings about the direct measurement of the analyte, normally in a comparatively short period of time (seconds or minutes). Any individual, suitably trained, could be capable of using such a device within a relatively short period. In environmental analysis, the low detection limits required make it essential to use sensitive techniques with low detection limits but which are simple and robust enough to be used in situ for field monitoring. Immunosensors fulfil some of these criteria. However, the requirement of speed and analytical simplicity cannot be attained with traditional heterogeneous assay formats.

Several groups have seen the need to eliminate the multiple steps in the assay to produce an effective biosensor format and various approaches have been applied [7–12]. These methods have been reviewed [13]. Although some of these systems have achieved the removal of the separation element of the heterogeneous immunoassay, they have achieved this at a penalty of increased assay complexity. This is far from ideal for commercial biosensor application. Killard et al. [14] have developed a very simple, genuine separation-free immunoassay system capable of the continuous monitoring of real-time biospecific interactions. The assay, although tested using a competition assay format is also applicable to sandwich assays. The assay is based on the principle of the 'electrically-wired' immunosensor developed by Lu et al. [15]. The basis of the biosensor is an antibody-modified polyaniline (PANI)/polyvinyl sulphonate (PVS) conductive polymer [16] that has been potentiodynamically assembled on the surface of a disposable carbon-paste electrode. Competition between horseradish peroxidase-labelled analyte and free analyte at the electrode surface brings about the reduction of hydrogen peroxide. Only conjugated material bound to the electrode surface via the antibody–antigen interaction brings about a catalytic reaction that is coupled to the electrode surface. Unbound material in the bulk solution does not induce any significant electrocatalytic current. The immunoassay format was shown capable of the competitive analysis of a test analyte, biotin using a traditional quasi-equilibrium assay format. Both batch analysis and flow-injection analysis were applied to the assay and found equal in terms of electrochemical responses. It was also demonstrated that in the flow-injection mode, the antibody–antigen interaction could be monitored continuously.

This paper extends the use of the biosensor system described above to the real-time analysis of antibody–antigen interactions to a batch system as well as a flow-injection system. It also illustrates the application of the biosensor for environmental analysis. Atrazine is a herbicide widely used by the agricultural industry to control broad leaf weeds in commercial crops and has been targeted under EU legislation as a drinking water contaminant, setting limits in potable water of 0.1 $\mu g\,l^{-1}$ [17]. Atrazine was measured using quasi-equilibrium and real-time batch analysis. Detection limits of 0.1 μM were achieved.

2. Experimental

2.1. Chemicals

Immunochemical reagents were purchased from Sigma–Aldrich (Poole, Dorset, UK). Anti-biotin antibody was a goat polyclonal (Sigma B3640), and horseradish peroxidase-labelled biotin (Biotin-HRP) was biotinamidocaproyl-labelled peroxidase (Sigma P2907). Horseradish peroxidase (HRP) was 200 U/mg (Sigma P 6782). Aniline was purchased from Aldrich (13,293-4), vacuum distilled and stored frozen under nitrogen. 30% hydrogen peroxide solution was purchased from Merck. Polyvilylsulphonate (PVS, 27,842-4) was purchased from Aldrich. Atrazine (12354 2H) was purchased from BDH. Atrazine was

covalently coupled to horseradish peroxidase according to Goodrow et al. [18]. Anti-atrazine antibody was prepared according to Fránek et al. [19].

2.2. Buffers and solutions

PBS is 0.1 M phosphate buffer, 0.137 M NaCl and 2.7 mM KCl, pH 6.8. This was prepared by mixing solutions containing 0.1 M Na_2HPO_4, 0.137 M NaCl and 2.7 mM KCl and 0.1 M KH_2PO_4 0.137 M NaCl and 2.7 mM KCl to a pH of 6.8. Unless otherwise stated, all immunochemicals were prepared in PBS.

2.3. Instrumentation

All electrochemical protocols were performed on a BAS100/W electrochemical analyser with BAS100/W software, using either cyclic voltammetry, or time-based amperometric modes.

2.4. Electrode preparation

2.4.1. Polymerisation of aniline on the electrode surface

Screen-printed carbon-paste electrodes were produced according to Cagnini et al. [20]. Electrodes were placed in 10 ml of 0.2 M H_2SO_4, prior to the polymerisation of aniline. A platinum mesh auxiliary and a silver/silver chloride reference electrode were used. Electrodes were cleaned and activated using cyclic voltammetry between −1200 and 1500 mV versus Ag/AgCl electrode at a scan rate of 100 mV/s, sensitivity of 1×10^{-3} A over one cycle.

A mixture of 7.8 ml 1 M HCl, 186 μl aniline (distilled and stored under nitrogen) and 2 ml polyvinylsulphonate (PVS) were degassed under nitrogen for 10 min. Aniline was polymerised on the surface of the working electrode using 10 voltammetric cycles between −500 and 1100 mV versus Ag/AgCl electrode at 100 mV/s, and sensitivity at 1×10^{-3} A.

2.4.2. Immobilisation of protein

Following polymerisation of aniline, the electrode was transferred to a 2 ml batch cell as previously described [14]. The surface of the polymer was reduced in 2 ml of PBS (degassed for 10 min under nitrogen) at −500 mV versus Ag/AgCl, sample interval of 500 ms, over 1500 s at a sensitivity of 1×10^{-4} A.

Antibody or enzyme was prepared in PBS prior to use. Very quickly after reduction was complete, PBS buffer was removed from the cell and quickly replaced with the protein solution, not under stirring or degassing. Again quickly, oxidation was performed at 700 mV versus Ag/AgCl for 1500 s.

During this oxidation, the protein becomes electrostatically attached to the polymer surface. Antibody or enzyme were carefully recovered from the cell and re-stored for later use.

2.5. Electroanalytical procedure

Electrodes that had been immobilised with antibody or enzyme were subjected to the appropriate immunochemical incubations as described in Section 2.6. Amperometric experiments were performed at −100 mV versus Ag/AgCl, with a sample interval of 500 ms and sensitivity of 1×10^{-4} A.

2.6. Immunoassay procedures

2.6.1. Protein binding capacity of the electrode surface

Solutions of HRP in doubling dilutions beginning at 10 mg/ml or solutions of anti-biotin antibody in doubling dilutions from 1 mg/ml were prepared and immobilised on the electrode surface according to Section 2.4.2. For electrodes modified with HRP, hydrogen peroxide at a concentration of 8 mM was added to the electrochemical cell containing 2 ml of PBS following the reaching of the steady state at −100 mV versus Ag/AgCl. For electrodes modified with anti-biotin antibodies, the electrode was incubated with biotin-HRP as described previously [14]. The electrode was transferred to PBS buffer followed by the addition of 8 mM hydrogen peroxide upon reaching the steady state.

2.6.2. Real-time monitoring of biotin-HRP-anti-biotin in a batch system

An electrode modified with anti-biotin antibody was placed in a 2 ml batch cell as previously described. PBS was added and the system stirred. Hydrogen peroxide was added to the cell at a concentration of 8 mM following reaching of the steady state. Concentrations of biotin-HRP between 0 and 25 μg/ml were added to the batch cell and the amperometric response monitored.

2.6.3. Quasi-equilibrium analysis of atrazine

Electrodes modified with 0.5 mg/ml anti-atrazine antibody were placed in a 2 ml batch cell. Incubation mixtures of 200 μl were added to the cell. These contained atrazine at a range of concentrations between 13.56 and 0.07 μM and atrazine-HRP at a concentration of 0.3 μg/ml. Incubation was performed at room temperature for 1 h. The incubation mixture was discarded and 2 ml PBS was added to the cell. Hydrogen peroxide at 40 mM was added to the cell upon reaching the steady state and the amperometric response monitored.

2.6.4. Real-time batch analysis of atrazine

Electrodes modified with 0.5 mg/ml anti-atrazine antibody were placed in the batch cell with 2 ml PBS. Following reaching of the steady state, 40 mM hydrogen peroxide was introduced at a concentration of 40 mM. Mixtures of atrazine-HRP (50 μg/ml) and atrazine between 0.93 and 29 mM were introduced to the stirred cell and the amperometric output monitored.

3. Results and discussion

3.1. Protein binding capacity of the polymer-modified electrode surface

In previous experiments [14], it was assumed that the modification of the electrode surface with antibody was best achieved by using a high protein concentration (1 mg/ml), so as to maximise the mass of protein on the electrode surface, as is the case with traditional ELISA assays. However, to assess the optimal protein binding capacity of the electrode surface, electrodes were modified with a range of concentrations of horseradish peroxidase (Fig. 1). This immediately illustrated that far from being optimal, the 1 mg/ml protein concentration yielded much lower catalytic signals than lower concentrations of enzyme. The optimum concentration was at approximately 0.5 mg/ml. However, very good signals could still be obtained at concentrations far below this. Signals at 0.01 mg/ml were comparable to 1 mg/ml in this regard. The same result was obtained when anti-biotin antibody was attached to the polymer backbone and visualised via interaction with biotin-HRP, demonstrating that essentially the mass of protein being bound to the surface is similar irrespective of the protein being used. It may also be the case that electron transfer at the electrode surface is more efficient at lower antibody concentrations.

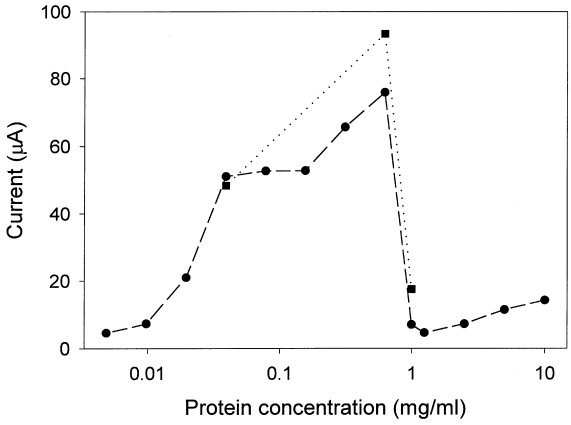

Fig. 1. Protein binding capacity of the polymer electrode surface. Concentrations of horseradish peroxidase above 1 mg/ml (dashed line) produced low amperometric responses. Below this, however, signals were greatly increased, with a maximum at approx. 0.5 mg/ml. A similar response was also noted when the system was tested with anti-biotin antibody and biotin-HRP (dotted line).

3.2. Real-time monitoring of antibody–antigen interactions in a batch system

Killard et al. [14] noted previously that monitoring real-time antibody–antigen interactions using this biosensor design was possible, simply with the presence of the enzyme-labelled analyte and the substrate hydrogen peroxide being present in solution. When applied to a flow cell configuration, no separation of bound and unbound species in the flow stream was required as only enzyme in association with the electrode surface via antibody–antigen interaction could yield a catalytic current. Fig. 2 illustrates the application of biotin-HRP to an anti-biotin-modified electrode in a 2 ml batch cell as previously described [14]. Upon addition of biotin-HRP to the cell containing buffer and substrate, binding of the biotin-HRP follows a typical rate curve, which is dependent, among other parameters, on the concentration of analyte. At higher tracer concentrations, signals were comparable to those previously seen for flow-injection. However, the batch system lost sensitivity at lower analyte

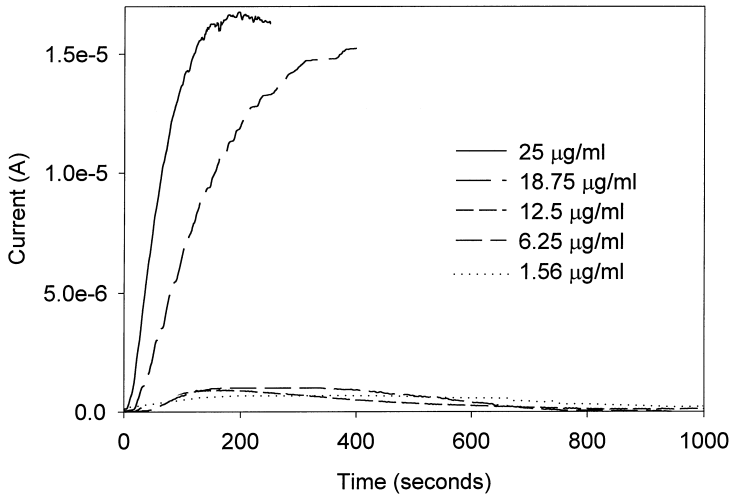

Fig. 2. Batch analysis of the real-time interaction between anti-biotin antibody and biotin-HRP. Higher concentrations of biotin-HRP brought about more rapid increases in current. The system was sensitive above 12.5 μg/ml. However, below this, it was difficult to differentiate between samples.

concentrations. It may be that mass transport limitation due to the large volume in the cell becomes more significant at these lower concentrations compared with the small cell volume and high linear velocities of the flow cell. Nevertheless, it was clear that real-time binding was possible using this very simple experimental set up.

3.3. Quasi-equilibrium analysis of atrazine

Previously, a competition immunoassay using this system had been performed using biotin as the model analyte [14]. However, the competition procedure had to be biased in favour of free biotin for effective competition to occur. This was assumed to be due to the characteristics of the anti-biotin polyclonal antibody used and not a flaw in the immunoanalytical procedure. To test this, the competition assay for atrazine was initially performed in a quasi-equilibrium format, allowing the labelled and unlabelled atrazine to bind to the surface of an electrode for 1 h. Table 1 shows that there is significant inter-electrode variability. This may be brought about by variations during printing, polymer deposition and immobilisation of the biological component and variability in the immunoanalytical procedure. This resulted in CVs between less than 3% and greater than 50%, although averaging at approximately 18%. Inter-electrode variability remains a

Table 1
Quasi-equilibrium analysis of atrazine

Atrazine concentration (μM)	Current (μA)	Standard deviation	Number of replicates	Coefficient of variation (%)
0	7.7	1.29	3	16.8
0.07	5.46	0.98	3	17.9
0.13	4.77	0.59	3	12.4
0.26	4.51	0.35	3	7.8
0.53	2.93	0.85	3	29.0
1.13	1.98	–	1	–
3.39	1.31	0.12	3	10.6
6.78	0.73	0.37	3	50.7
13.56	0.33	<0.01	3	<3.0

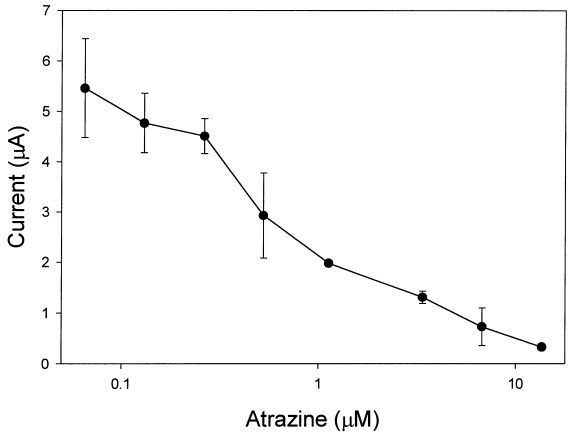

Fig. 3. Quasi-equilibrium analysis of atrazine. Binding of atrazine in the competition assay format used followed a typical titration curve in a range beyond 0.1–10 μM. The assay was most sensitive between 0.3 and 1 μM atrazine, with detection down to 0.13 μM.

significant problem with this system, as each electrode is only capable of generating a single data point and many electrodes must be prepared to run a single standard curve. However, effective competition was apparent, even below an atrazine concentration of 0.1 μM, with an effective assay range between 0.1 and 10 μM (Fig. 3). The assay was most sensitive between 0.3 and 1 μM atrazine. Using paired and independent t-tests it was also shown that detection was possible to 0.13 μM (28 ppb) (p=0.95). Improvements in inter-electrode variability and analysis at lower concentration levels may yield better detection limits. Presently, other systems can measure atrazine at and beyond the EU limit of 0.1 $\mu g\,l^{-1}$ (0.1 ppb) [21]. As the assay had taken place under free competition, it was clear that previous problems experienced in generating a competitive assay [14] were a result of the particular antibody being used. The monoclonal antibody being used here has been previously characterised [22] and shown to have an excellent binding affinity of $3.08\times10^{8}\,M^{-1}$, which is essential for the establishment of a sensitive immunosensor. The selectivity of the system is also dependent on the selectivity of the antibody being used.

3.4. Real-time batch analysis of atrazine

The ability of the biosensor to measure atrazine in real time using a batch system was assessed. Free competition between labelled and unlabelled atrazine was allowed to take place in the batch cell in the presence of substrate. A series of rate curves were produced typical of those in Fig. 2. Graphs were constructed based on the resulting maximum current

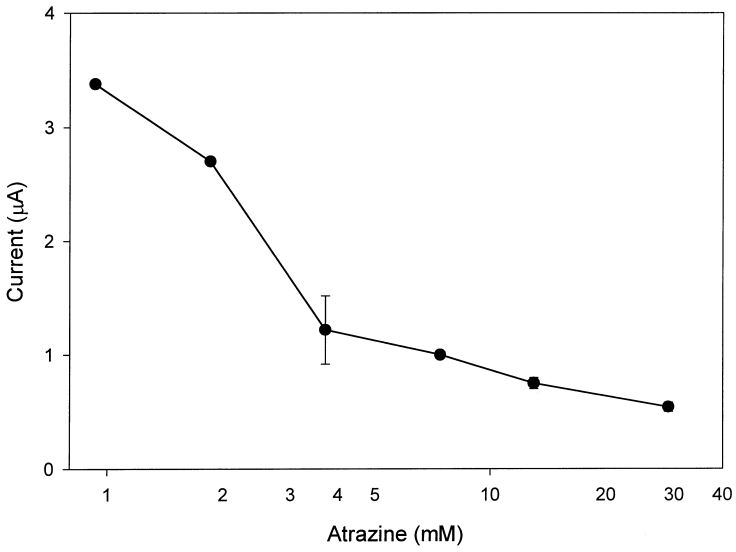

Fig. 4. Analysis of atrazine in real-time using maximum response data. The assay was performed between 30 and 1 mM. Increased sensitivity was seen below 4 mM.

Table 2
Maximum response data for the real-time stirred batch analysis of atrazine

Atrazine concentration (μM)	Current (μA)	Standard deviation	Number of replicates	Coefficient of variation (%)
0	17.66	1.26	3	7.1
0.93	3.38	±0.16	2	–
1.85	2.70	±0.24	2	–
3.7	1.22	0.30	3	24.6
7.4	1.00	0.03	3	3.0
13	0.75	0.05	3	6.7
29	0.54	0.04	3	7.4

responses and on the initial current responses within the first 10–30 s. Using maximum current response data (Fig. 4 and Table 2), it can be seen that a relationship exists between atrazine concentration and maximum current responses between 1 and 10 mM. This is some three orders of magnitude less sensitive than the quasi-equilibrium incubation. However, this is not surprising because of the comparatively large cell volume and the mass transport limitations that result. When analysed kinetically (Fig. 5), improved sensitivity was apparent below 4 μM. Except for a single datum (Table 2), inter-electrode variability remained beneath 8%. This was a significant improvement over quasi-equilibrium analysis and might suggest that significant variability may be introduced as a result of the immunoanalytical procedure. In the quasi-equilibrium methodology, incubation of a 200 μl sample takes place in a static batch cell and may encounter variations such as evaporation, causing changes in concentration. In addition, the method relies on diffusion, whereas the batch system introduces stirring and so maintains homogeneity.

In its present form, this system does not meet legislative requirements for the detection of atrazine. However, it does demonstrate proof of principle that a very simple immunosensor format is appropriate for the analysis of such. In addition, diffusion-limited, flow-injection and batch systems have been shown

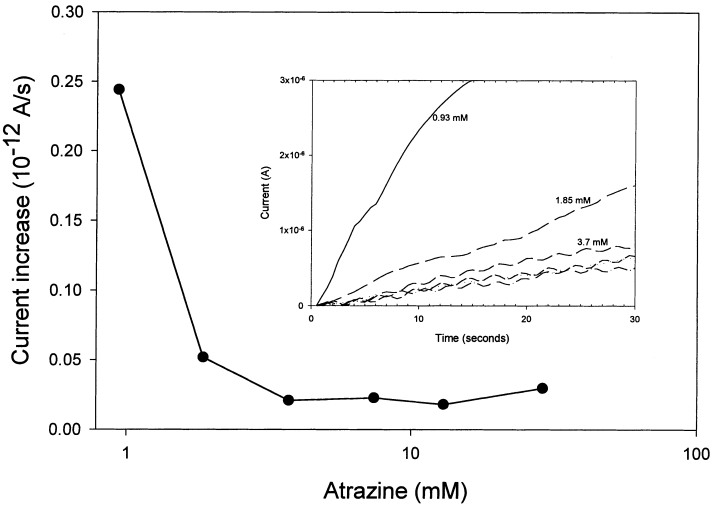

Fig. 5. Analysis of atrazine in real-time using kinetic data. The insert shows that current increases in the first 10–30 s were linear for all samples. When the rates of current change were calculated, the assay was seen to be sensitive below 4 mM. Above this, no significant differences in current response were apparent.

capable of these analyses. Further studies at lower analytical ranges may illustrate whether greater limits of detection may be achieved.

In its present form, the system is most suited towards single use, disposable applications, as methods of surface regeneration have not yet been explored. Such regenerability and reusability would be an essential element of any system required for long-term, in situ environmental monitoring.

4. Conclusions

The immunosensor described has the ability to be used for the analysis of haptens such as the pesticide atrazine using a competition immunoassay format. It was also illustrated that, as well as being applicable in flow-injection mode, the system could also be used in batch and quasi-equilibrium set-ups. The system has been shown capable of detection of atrazine at 0.13 μM (28 ppb). The lower limit of the assay was not encountered and the assay may be further enhanced by the use of a flow system in which mass transport conditions would be significantly improved. This may bring it within reach of standards required for environmental monitoring. All this could be performed in a single-step immunoassay format with a minimal number of reagents. This system has the potential to form the basis of many facile measurements not previously amenable to biosensor analysis.

Acknowledgements

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References

[1] H. Huang, M.M. Garcia, B.W. Brooks, K. Nielsen, S.-P. Ng, Int. J. Food Microbiol. 51 (1999) 85.
[2] W. Zingg, W. Bossart, E. Berli, D. Nada, J. Virol. Meth. 79 (1999) 141.
[3] M. Fránek, V. Pouzar, V. Kolár, Anal. Chim. Acta 347 (1997) 163.
[4] C.M. Westhoff, O. Lopez, P. Goebel, L. Carlson, R.R. Carlson, F.W. Wagner, S.M. Schuster, D.E. Wylie, Proteins 37 (1997) 429.
[5] A.J. Killard, B. Deasey, R. O'Kennedy, M.R. Smyth, Trends Anal. Chem. 14 (1995) 257.
[6] P. Tijssen, Practice and Theory of Enzyme Immunoassay, Elsevier, Oxford, 1985.
[7] C. Duan, M.E. Meyerhoff, Anal. Chem. 66 (1994) 1369.
[8] A.P. Osipov, N.V. Zaitseva, A.M. Egorov, Biosens. Bioelectron. 11 (1996) 881.
[9] D. Ivnitski, J. Rishpon, Biosens. Bioelectron. 11 (1996) 409.
[10] J. Rishpon, D. Ivnitski, Biosens. Bioelectron. 12 (1997) 195.
[11] D. Ivnitski, T. Wolf, B. Solomon, G. Fleminger, J. Rishpon, Bioelectrochem. Bioeng. 45 (1998) 27.
[12] R.W. Keay, C.J. McNeil, Biosens. Bioelectron. 13 (1998) 963.
[13] A.J. Killard, M.R. Smyth, Anal. Lett. 33 (2000) 1451.
[14] A.J. Killard, S. Zhang, H. Zhao, R. John, E.I. Iwuoha, M.R. Smyth, Anal. Chim. Acta 400 (2000) 109.
[15] B. Lu, E.I. Iwuoha, M.R. Smyth, R. O'Kennedy, Anal. Chim. Acta 345 (1997) 59.
[16] E.I. Iwuoha, D. Saenz de Villaverde, N.P. Garcia, M.R. Smyth, J.M. Pingarron, Biosens. Bioelectron. 12 (1997) 749.
[17] 80/778/EC, Council Directive relating to the quality of water intended for human consumption, Off. J. Eur. Commun. L229 (1980) 11–49.
[18] M.H. Goodrow, R.O. Harrison, B.D. Hammock, J. Agric. Food Chem. 38 (1990) 990.
[19] M. Fránek, V. Kolár, M. Granátová, Z. Nevoránková, J. Agric. Food Chem. 42 (1994) 1369.
[20] A. Cagnini, I. Palchetti, I. Lionti, M. Mascini, A.P.F. Turner, Microchim. Acta 121 (1995) 85.
[21] M. Fránek, V. Kolár, S.A. Eremin, Anal. Chim. Acta 311 (1995) 349.
[22] C. Steegborn, P. Skládal, Biosens. Bioelectron. 12 (1997) 19.