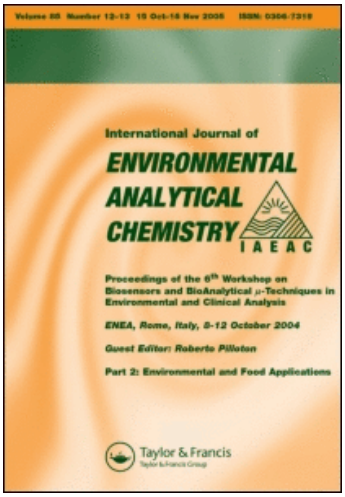


International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

# Gold nanoparticle-based immunochromatographic assay for the detection of 7-aminoclonazepam in urine

Qiusheng Li[a]; Liqiang Liu[a]; Wei Chen[a]; Chifang Peng[a]; Libing Wang[a]; Chuanlai Xu[a]
[a] School of Food Science and Technology, Jiangnan University, Wuxi, JiangSu, People's Republic of China

**To cite this Article** Li, Qiusheng , Liu, Liqiang , Chen, Wei , Peng, Chifang , Wang, Libing and Xu, Chuanlai(2009) 'Gold nanoparticle-based immunochromatographic assay for the detection of 7-aminoclonazepam in urine', International Journal of Environmental Analytical Chemistry, 89: 4, 261 — 268
**To link to this Article: DOI:** 10.1080/03067310802538493
**URL:** http://dx.doi.org/10.1080/03067310802538493

*Intern. J. Environ. Anal. Chem.*
Vol. 89, No. 4, 10 April 2009, 261–268

Taylor & Francis
Taylor & Francis Group

# Gold nanoparticle-based immunochromatographic assay for the detection of 7-aminoclonazepam in urine

Qiusheng Li, Liqiang Liu, Wei Chen, Chifang Peng, Libing Wang and Chuanlai Xu*

*School of Food Science and Technology, Jiangnan University, Wuxi, JiangSu, People's Republic of China*

(*Received 21 June 2008; final version received 7 October 2008*)

An analytical system of immunochromatographic assay based on gold nanoparticles was developed for the detection of 7-aminoclonazepam (7-ACLZ) in human urine. The qualitative assay was based on the competitive immunoassay using anti-7-ACLZ polyclonal antibody (PcAb) and a detector reagent that contains colloidal gold particles coated with anti-7-ACLZ PcAb. Nitrocellulose membrane was separately immobilised with goat anti-rabbit IgG (control line) and 7-ACLZ-OVA conjugate (test line). The sensitivity of the strip was tested for detecting 7-ACLZ spiked in urine and each specimen was independently measured by liquid chromatography tandem mass spectrometry. Good correlation was showed by the recovery results. The limit of detection for the strip test in urine was 100 ng $mL^{-1}$. The assay can be applied to the rapid detection of 7-ACLZ with the short testing time.

**Keywords:** 7-aminoclonazepam; lateral flow; gold-labelled immunochromatography; LC/MS/MS



## 1. Introduction

Benzodiazepine is a kind of psychotropic drugs, which can eliminate erethism, manic anxiety. Benzodiazepines can make human into a quiet and somnolent state by selectively inhibiting unrest and tension. In general, benzodiazepines act as hypnotics in high doses, anxiolytics in moderate doses, and sedatives in low doses [1–4]. In China, with the ever increasing work pressure, the situation of young people suffering from insomnia is more and more serious. Benzodiazepines are mainly used for the treatment of insomnia in clinics. Clonazepam is a benzodiazepine derivative. The 7-aminoclonazepam (7-ACLZ) is the major urinary metabolite (target metabolite) of clonazepam [5–7]. For screening large numbers of samples, it is significant for developing a rapid, simple assay for detecting 7-ACLZ in clinical cases.

A number of methods are available for benzodiazepines detection including immunoassay and chromatographic methods. A range of immunoassay procedures using enzyme immunoassay, fluorescent immunoassay, kinetic interaction of microparticles immunoassay, cloned enzyme donor immunoassay, radio immunoassay and enzyme-linked immunosorbent assay are now available. Chromatographic methods using high-performance liquid chromatography (HPLC), liquid chromatography tandem

*Corresponding author. Email: xcl@jiangnan.edu.cn

ISSN 0306–7319 print/ISSN 1029–0397 online

DOI: 10.1080/03067310802538493
http://www.informaworld.com

mass spectrometry (LC/MS/MS), gas chromatography and gas chromatography tandem mass spectrometry methods had been reported [8–11].

The lateral flow strip is one kind of immunochromatographic assay depending on the transport of a reactant to its binding partner immobilised on the surfaces of the membrane [12–14]. It is based on an immunochromatographic procedure that utilises antigen–antibody properties in a novel manner and provides rapid detection of analyte. For a smaller analyte (hapten), especially drugs of abuse and steroid-based ovulation prediction, the competitive format assay is mainly used [15,16]. In this type of assay, the detector reagent is typically colloidal gold-labelled antibodies against the analyte. The capture line is normally analytes conjugated to a carrier protein immobilised on the membrane. Analytes in samples will compete with analytes immobilised on the membrane to bind to the detector antibody. This technique includes four benefits of user-friendly format, very short time to test, long-term stability over a wide range of climates and relatively inexpensive to make. These characteristics render it ideally suited for on-site testing by untrained personnel. There are a lot of commercial immunochromatographic tests for benzodiazepines, but the cross-reactivities of the tests indicated that they cannot be applied to analyse the metabolite (7-ACLZ). In this study, we describe the development of one-step colloidal gold-based immunochromatographic assays for the detection of 7-ACLZ.



## 2. Experimental

### 2.1 *Materials and chemicals*

7-Aminoclonzepam, 7-aminonitrazepam (7-ANIZ) and other benzodiazepines used in the specificity test were purchased from Cerilliant. Chloroauric acid, goat anti-rabbit IgG and other chemicals were purchased from Sigma (St. Louis, USA). Bovine serum albumin (BSA), ovalbumin (OVA), and a protein-A-agarose affinity chromatography column were purchased from the Sino-American Biotechnology Company, imported in bulk. Nitrocellulose hi-flow plus membrane and glass fibre membrane were purchased from Schleicher & Schuell Gmbh (Dassel, Germany). Semi-rigid polyethylene sheets and adhesive tape were purchased from the local market. Filter paper and analytical grade buffer chemicals were purchased from Boao Biotech Co., Ltd. Shanghai, China. Ultrapure water was manufactured by the Milli-Q Ultra Pure System (Millipore, Bedford, MA). Buffers were prepared in our lab. All other chemicals used in this study were of analytical grade or higher.

### 2.2 *Preparation of polyclonal antibody to 7-ACZP*

7-Aminoclonazepam–BSA conjugation was synthesised by diazotization [17], 7-ACLZ–OVA was synthesised in the same way. The polyclonal antibody (PcAb) specific for 7-ACLZ was obtained by immunising New Zealand female white rabbits with 7-ACLZ–BSA. Antibodies were purified by ammonium sulfate precipitation followed by further purification using a protein-A-agarose affinity chromatography column.

### 2.3 *Preparation of gold nanoparticles*

The 20 nm gold nanoparticles were prepared by a modified citrate reduction method [18]. Briefly, 200 mL of 0.01% $HAuCl_4$ solution (in Milli-Q purified water) was boiled

thoroughly, and then 2.6 mL of 1% sodium citrate solution was added rapidly under constant stirring. After the colour of the solution had changed from pale yellow to burgundy within 2 min, it was boiled for another 15 min. After the solution reached room temperature, it was filtered through a 0.8 μm Gelman membrane filter. The colloidal gold obtained was scanned by UV–vis spectroscopy between 200 and 700 nm, showing there was only one maximum absorbent wavelength; 521 nm. With the measurement by transmission electron microscope (TEM), the average diameter of these particles of uniform size was 20 nm.

### 2.4 *Preparation of gold nanoparticle-conjugated antibodies*

The pH of colloidal gold solution for anti-7-ACLZ PcAb conjugation was adjusted to pH 9.0 with 0.1M $K_2CO_3$. Before conjugation, the optimal concentration of antibody for conjugation was determined by UV–vis spectroscopy. Ten portions of 1 mL of purified anti-7-ACLZ PcAbs that were diluted into gradient concentrations (from 2 to 20 g $mL^{-1}$) were added into 1 mL of colloidal gold solutions. The mixtures were incubated for 15 min at RT, and then, 0.1 mL of 10% (w/v) NaCl solution was added. A curve of the concentration and the absorbance was obtained by detecting the absorbances of the mixtures at 520 nm. The protein concentration at the point closest to abscissa in the graph was considered as optimum with a 20% increment. It was found that 6g was the best results. A 0.03 mL aliquot of purified anti-7-ACLZ PcAb (0.2 mg $mL^{-1}$) was added to the colloidal solution (1 mL) and stirred gently at 4°C for 20 min. The conjugate was stabilised with 10% BSA and adjusted to a final concentration of 1%. The mixture was incubated for 1 h at room temperature and centrifuged for 15 min at 9000 *g*. The supernatant was discarded and the pellet was resuspended in 0.002M, pH 9.0 borate buffer (BB) containing 0.05% Triton X-100 and 5% cane sugar. This step was repeated twice. The concentrated conjugate was stored at 4°C until required for use.



### 2.5 *Preparation of the lateral flow strip*

The sample pad, which influences the migration of the gold nanoparticles–antibody conjugate, was treated with 0.01M, pH 7.4, phosphate buffered saline (PBS) containing

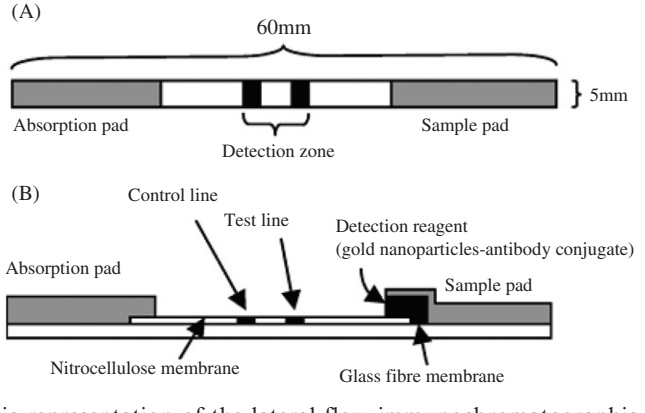


Figure 1. Schematic representation of the lateral flow immunochromatographic test strip. (A) top view; (B) cross section.

0.5% BSA and 0.05% sodium azide. 7-ACLZ–OVA diluted to 1 mg mL$^{-1}$ with 0.01M, pH 7.4, PBS containing 3% methanol was used as the test line (T) capture reagent, while goat anti-rabbit IgG diluted to 0.5 mg mL$^{-1}$ with 0.01M PBS, pH 7.4 was used as the control line (C) capture reagent. These capture reagents were dispensed by the BioJet Quanti3000$^{TM}$ dispenser onto a nitrocellulose membrane as the test and control lines. The gold-labelled 7-ACLZ PcAb was dispensed by AirJet Quanti3000$^{TM}$ onto a glass fibre membrane which was called the conjugate pad. After drying the membranes for 1 h at room temperature, the nitrocellulose membrane, conjugate pad, sample pad and absorbent pad were laminated and pasted onto a plastic backing plate. Then the card was cut into 5 mm-wide strips (Figure 1) using a strip cutter model CM4000. Then the strips were stored in a self-seal plastic bag with desiccant at 4°C.

### 2.6 *Preparation of spiked urine samples*

Bland urine samples were obtained from drug free volunteers. Samples were filtered through a Whatman no. 1 filter paper, and stored at −20°C in aliquots of 10 mL for further experiment. Then urine samples were serially spiked with 7-ACLZ to make solutions at 0, 5, 20, 50, 100 and 200 ng mL$^{-1}$ concentration levels prior to lateral flow test [19,20].



### 2.7 *Real sample analysis*

Three volunteers were asked to take a dose of NZP, 2 mg L$^{-1}$ (therapeutical dose: 1–2 mg L$^{-1}$). Blank urines were collected before taking drug, and urines were collected 4, 12, 20, 32 and 48 h after drugs were taken.

### 2.8 *Analysis by LC/MS/MS*

To perform a confirmation analysis by LC/MS/MS, 7-ANIZ (internal standard) was added to the samples that were liquid–liquid extracted at pH 10.8, filtration with 0. 45 μm membrane before analysed. The qualitative analysis was based on the retention time, and the quantitation was carried out with intension of the internal standard. LC/MS/MS was carried out under the conditions as follows:

Chromatographic conditions: chromatographic separation was performed on a Varian Polaris $C_{18}$-A column (5 μm, 2.1 mm × 150 mm) at 50°C. A 20 μL aliquot of the extract was injected for HPLC analysis. The flow rate was 0.2 mL min$^{-1}$. The mobile phase was composed of water (solvent A) and ACN (solvent B). The time programme for the multi-step gradient was: 0 min, 20% solvent A; 4.0 min, 90% solvent A; 7.0 min, 90% solvent A; 7.1 min, 20% solvent A; 9 min, 20% solvent A; loop time = 9.0 min.

Mass spectrometry: the instrument was operated in the positive ionisation mode. Best results were obtained with a spray voltage of 4.5 kV, capillary temperature of 350°C, the sheath and auxiliary gas pressures (nitrogen) set at 30 and 5 (arbitrary units), respectively. Mass spectral acquisition was applied with selective reaction monitoring of two diagnostic transition reactions. Data were recorded in the selected reaction monitoring (SRM) acquisition mode.

### 2.9 *Procedure of lateral flow strip*

The samples for detection were added into several duplicates of the 96-well microtitre plate, each well of 100 μL. The end of the sample pad of the test strip was inserted into the well for 10 s, while the sample pad was rapidly drenched and the conjugate pad on which colloidal gold labelled anti-7-ACLZ PcAb reagent was solubilised began to move accompanying the sample flow front-up the NC membrane. The results of the two red lines can be judged effectively by naked eyes. And if no line emerges, the test is invalid. The total assay time was less than 5 min.

## 3. Results and discussion

### 3.1 *Detection limit of the test strip*

The resulting gold nanoparticles were determined by measuring the size of the particle by TEM. The particles had sizes of approximately 20 nm in Figure 2. In the detection zone, the negative result was judged by the appearance of two red lines at control and test region, and the positive result was judged by the appearance of only a single line in the control region. The test is invalid if no line is present in the test strip or only one line appears in the test region. The intensity of the test line is proportional to the amount of 7-ACLZ present in the sample. The more 7-ACLZ present in the sample, the weaker the test line appears. As shown in Figure 3, 50 ng mL$^{-1}$ of 7-ACLZ spiked solution caused a slight but distinguishable difference compared to the negative control for lateral-flow assay. On the basis of complete disappearance of colouration of the analytical line, 100 ng mL$^{-1}$ of 7-ACLZ was considered to be the detection limit for lateral flow assay. In addition, it is possible to decrease the limits of detection (LOD) by adding sensitizer and optimising the relevant parameters [21].



### 3.2 *Specificity of lateral flow strip*

The related benzodiazepines compounds were prepared in the same way as 7-ACLZ. Then these standard compounds were examined and judged using the naked eye. The cut-off

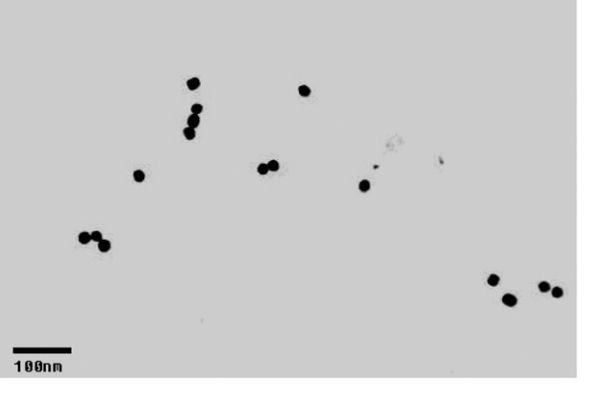


Figure 2. TEM photographs of gold particles of sizes produced using sodium citrate reduction of solutions of $HAuCl_4$.

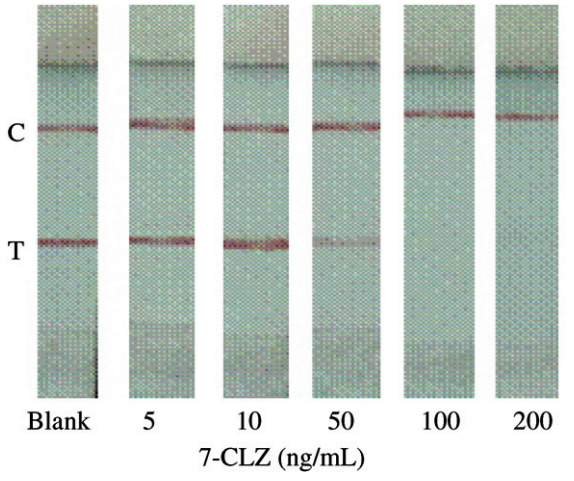


Figure 3. Lateral flow stirp test of 7-ACLZ. Upper line: control line (goat anti-rabbit IgG); lower line: test line (7-ACLZ–OVA). 7-ACLZ concentration from left to right: 0, 5, 20, 50, 100 and 200 ng $mL^{-1}$. The colour intensity of 50 ng $mL^{-1}$ was clearly distinguishable from that of the negative control.



Table 1. The cut-off limit value of 7-aminoclonazepam and related benzodiazepines compounds in human urine by flow strip assay.

| Substances | CAS-No. | Cut-off limit value (ng $mL^{-1}$) |
|---|---|---|
| 7-Aminoclonazepam | – | 100 |
| Clonazepam | 1622-61-3 | 100 |
| 7-Aminonitrazepam | – | 100 |
| Nitrazepam | 146-22-5 | 200 |
| Flunitrazepam | 1622-62-4 | 500 |
| Nordazepam | – | 500 |
| Diazepam | 439-14-5 | 500 |
| Oxazepam | 604-75-1 | 500 |
| Lorazepam | 864-49-1 | 1000 |
| Chlordiazepoxide | 58-25-3 | 25,000 |
| Triazolam | 28911-01-5 | 30,000 |

limit concentrations of 7-ACLZ and related benzodiazepines compounds in human urine by flow strip assay are shown in Table 1. The results indicated that the strip assay was class-selective towards 7-ACLZ, clonazepam, 7-ANIZ and nitrazepam, there was little cross-reactivity with other analogues. The immunochromatographic tests for benzodiazepines had been investigated by many laboratories and companies. However, there are many disadvantages of their products. For instance, the results of cross-reactivity of ACON Laboratories indicated that the antibody they used was class-selective towards oxazepam (100%), alprazolam (153%), clobazam (306%), chlorazepate (154%) and nitrazepam (306%), but just had little cross-reactivity with clonazepam (36%) and no investigation of 7-ACLZ and 7-ANIZ. Furthermore, the LOD of the products

Table 2. Results confirmation of flow strip test on human urine by LC-MS/MS.

| Spiked level ($ng\,mL^{-1}$) | Visual results[a] ($n=3$) | Results of LC-MS/MS ($ng\,mL^{-1}$), mean ± SD ($n=3$) |
|---|---|---|
| 5 | −, −, − | 0.39 ± 0.16 |
| 20 | −, −, − | 18.7 ± 0.5 |
| 50 | −, −, − | 49.1 ± 0.72 |
| 100 | ±, ±, ± | 98.9 ± 1.45 |
| 200 | +, +, + | 198.5 ± 1.1 |

Note: [a]+, positive, 7-ACLZ concentration was more than $100\,ng\,mL^{-1}$; −, negative, 7-ACLZ concentration was less than $100\,ng\,mL^{-1}$; ±, positive/negative, 7-ACLZ concentration was around $100\,ng\,mL^{-1}$.

Table 3. Results of 7-ACLZ in urine of volunteers taking 2 mg of clonazepam.

| Volunteers | Flow strip assay | | | | | LC-MS/MS ($ng\,mL^{-1}$) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Sex/age | 4 h | 12 h | 20 h | 32 h | 48 h | 4 h | 12 h | 20 h | 32 h | 48 h |
| Man/25 | − | − | + | ± | − | 42 | 71 | 230 | 95 | 70 |
| Man/28 | − | − | ± | − | − | 50 | 70 | 108 | 80 | 35 |
| Women/28 | − | − | ± | + | − | 45 | 78 | 105 | 185 | 81 |

Note: [a]+, positive, 7-ACLZ concentration was more than $100\,ng\,mL^{-1}$; −, negative, 7-ACLZ concentration was less than $100\,ng\,mL^{-1}$; ±, positive/negative, 7-ACLZ concentration was around $100\,ng\,mL^{-1}$.



(ACON Laboratories, Medimpex United company and ALL.DIAG company) were high ($300\,ng\,mL^{-1}$) compared with our results ($100\,ng\,mL^{-1}$).

### 3.3 *Reliability of the test strip assay and analysis by LC/MS/MS*

The reliability of the immunochromatographic assay was determined by carrying out the test with the uncontaminated samples spiked with 7-ACLZ concentrations at 5, 20, 50, 100 and $200\,ng\,mL^{-1}$, and analysed by immunoassay and LC/MS/MS. The samples were screened with gold-based immunoassay and confirmed by LC/MS/MS. The results shown in Table 2 indicated that the correlation between the two methods was good. Additionally, after storing at 37°C for 7 days, due to the stability of gold as labelling agent, this strip also had a better effect than enzyme immunoassay test strip, that the urine samples, enzyme-labelled analyte and a nitrocellulose test strip, containing immobilised antibodies, are incubated together, after which the strip is placed in a chromogen-containing substrate solution for colour reaction [22].

### 3.4 *Real sample analysis*

The urines samples of the three volunteers were collected at different time and analysed by the immunochromatographic method that we developed, and quantified by LC/MS/MS. The results were shown in Table 3 which indicated that the method was reliable and can be applied to the detection of 7-ACLZ in urine sample.

## 4. Conclusions

There are many advantages of the lateral flow strip assays: the testing process requires no equipment; the results are obtained in only 5 min and with simple sample pretreatment. So, these assays could be used as convenient qualitative tools for the rapid screening of 7-ACLZ in urine. However, the method provides only a preliminary, semi-quantitative result. The result could be used to judge whether the 7-ACLZ concentration remaining in the sample was higher than the detection limit or not and a quantitative result of samples could be further obtained by LC/MS/MS.

## Acknowledgement

We greatly appreciate the financial support from National Natural Science Foundation of China (20675035).

## References

[1] L.E. Hollister, B. Müller-Oerlinghausen, K. Rickels, and R.I. Shader, J. Clin. Psychopharmacol **13**, 1 (1993).
[2] H. Ashton, Drugs **48**, 25 (1994).
[3] L. Bailey, M. Ward, and M.N. Musa, J. Clin. Pharmaco. **34**, 804 (1994).
[4] N.S. Miller and M.S. Gold, J. Subst, Abuse Treat. **8**, 3 (1991).
[5] R.C. Baselt and R.H. Cravey, *Disposition of Toxic Drugs and Chemicals in Man*, 4th ed. (Biomedical Publications, Foster City, CA, 1995).
[6] S.J. Salamone, *Benzodiazepines and GHB: Detection and Pharmacology* (Humana Press, Totowa, NJ, 2001).
[7] M.J. LeBeau, Forensic Sci. **44**, 227 (1999).
[8] O.H. Drummer, J. Chromatogr. B. **713**, 201 (1998).
[9] A. Negrusz, A.M. Bowen, C.M. Moore, S.M. Dowd, M.J. Strong, and P.G. Janicak, Anal. Bioanal. Chem. **376**, 1198 (2003).
[10] X.P. Lee, T. Kumazawa, J. Sato, Y. Shoji, C. Hasegawa, C. Karibe, T. Arinobu, H. Seno, and K. Sato, Anal. Chim. Acta **492**, 223 (2003).
[11] L.P. Wang, H.X. Zhao, Y.M. Qiu, and Z.Q. Zhou, J. Chromatogr. A **1136**, 99 (2006).
[12] A.Y. Kolosova, L. Sibanda, F. Dumoulin, J. Lewis, E. Duveiller, C. Van Peteghem, and S. De Saeger, Anal. Chim. Acta **616**, 235 (2008).
[13] R.J. Cui, H.P. Huang, Z.Z. Yin, D. Gao, and J.J. Zhu, Biosens. Bioelectron. **23**, 1666 (2008).
[14] C. Zhang, Y. Zhang, and S. Wang, J. Agric. Food Chem. **54**, 2502 (2006).
[15] A. Roda, A.C. Manetta, O. Portanti, M. Mirasoli, M. Guardigli, and P. Pasini, Luminescence **18**, 72 (2003).
[16] S. Qian and H.H. Bau, Anal. Biochem. **326**, 211 (2004).
[17] D. Ross, Method. Enzymol. **84**, 492 (1982).
[18] K.C. Grabar, R.G. Freeman, M.B. Hommer, and M.J. Natan, Anal. Chem. **67**, 735 (1995).
[19] P. Andrzej, Z. Jan, and N. Jolanta, Anal. Chim. Acta **483**, 61 (2003).
[20] G.P. Zhang, X.N. Wang, J.F. Yang, Y.Y. Yang, G.X. Xing, Q.M. Li, D. Zhao, S.J. Chai, and J.Q. Guo, J. Immunol. Method. **312**, 27 (2006).
[21] N. Nagatani, R. Tanaka, T. Yuhi, T. Endo, K. Kerman, Y. Takamura, and E. Tamiya, Sci. Technol. Adv. Mater. **7**, 270 (2006).
[22] M.E. Ploum, W. Haasnoot, R.J. Paulussen, G.D. van Bruchem, A.R. Hamers, R. Schilt, and F.A. Huf, J. Chromatogr. **564**, 413 (1991).