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Determination of ascorbic acid in individual rat hepatocyte by capillary electrophoresis with electrochemical detection

Xuemei Sun, Yan Niu, Sai Bi, Shusheng Zhang*

Key Laboratory of Eco-chemical Engineering, Ministry of Education, College of Chemistry and Molecular Engineering, Qingdao University of Science and Technology, Qingdao 266042, PR China

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ABSTRACT

A method for the direct determination of ascorbic acid (AA) in individual rat hepatocyte based on capillary electrophoresis (CE) coupled with electrochemical detection (ECD) using a new kind of homemade carbon fiber micro-disk bundle electrode has been described. Individual rat hepatocytes were injected into a fused-silica capillary with an inner diameter of 25 μm, and lysed by 0.1% sodium dodecylsulfate (SDS) as cell lysis solution. The following conditions were suitable for the determination of AA: running buffer, 1.83×10^{-2} mol/l Na_2HPO_4–1.70×10^{-3} mol/l NaH_2PO_4 (pH 7.8); separation voltage, 20.0 kV; detection potential, 0.80 V (*vs.* saturated calomel electrode (SCE)). The concentration limit of detection (LOD) of the method was1.7×10^{-6} mol/l at a signal-to-noise (*S/N*) ratio of 3, and the mass LOD was 3.0 fmol. The linear dynamic range was from 5.0×10^{-6} to 5.0×10^{-4} mol/l with a correlation coefficient of 0.9962 for the injection voltage of 5.0 kV and injection time of 10 s. The relative standard deviation (R.S.D.) was 0.85% for the migration time and 1.8% for the peak current. This method was successfully applied to AA determination in rat hepatocyte. The recovery was between 91% and 97%, and the amount of AA in single rat hepatocyte ranged from 28 to 63 fmol.

1. Introduction

Ascorbic acid (AA) is one of the most important cellular antioxidants [1,2] and a valuable biomarker of oxidative stress [3–5]. It has been found that AA can inhibit viral infectivity by inactivating viruses and by affecting viral replication. At the cellular level, AA can mitigate the reactive oxygen species production triggered by Lipopolysaccharide and thereby prevents the induction of nitric oxide synthase and excessive production of nitric oxide that worsens oxidative stress in hepatocytes [6,7]. Additionally, AA acts through redox-sensitive signaling pathways to induce tolerance in the dendritic cells of the immune system [8]. It has also been found that AA can suppress human immunodeficiency virus (HIV) expression [9]. Therefore, it is very important to develop a rapid, simple, sensitive and accurate method for the determination of AA.

Single-cell analysis is an interesting and significant technique in which the difference between cells in chemical composition, biological activity, and the responses to external stimuli can be discerned [10,11]. Capillary electrophoresis (CE) with electrochemical detection (ECD) has already been applied to analyse single cells because it can offer high sensitivity and selectivity for electroactive analytes [12–16]. Working electrode is very important in the development of CE–ECD. Many kinds of working electrodes have been used, such as platinum electrode [17,18], carbon fiber bundle-Au/Hg dual electrode [19], gold/mercury amalgam microelectrode [20], gold electrode [21], carbon disk electrode [22], and carbon fiber micro-disk bundle electrode (CFMBE) [23–25]. Among them, CFMBE has widely been used to detect alkaline phosphatase isoenzymes in individual mouse bone marrow fibroblast cells [12], AA in single human neutrophils [14], Glucose-6-phosphate dehydrogenase in individual human erythrocytes [16], lactate dehydrogenase in human erythrocytes [26], free intracellular amino acids in single mouse peritoneal macrophages [15] etc., because of its high sensitivity and stability.

In this paper, a new kind of homemade CFMBE was applied to determine AA in individual rat hepatocytes by CE–ECD. Compared with the CFMBE we previously used which was the same as the one described in Jin's paper [23], the new one was more friendly to the environment because it was free from mercury, and it was simple in fabrication procedure and convenient in detection. To the best of our knowledge, this work represents the first demonstration of CE–ECD used for single-cell analysis of AA in individual rat hepatocyte cells. The results showed that this method is a simple, sensitive and reliable technique.

* Corresponding author. Fax: +86 532 84022750.
E-mail address: shushzhang@126.com (S. Zhang).

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2. Experimental

2.1. Apparatus and equipments

The CE separation system and the arrangement of the ECD cell used in this work were similar to that described in Jin's paper [27]. Briefly, a reversible high-voltage power supply (Model 9323-HVPS; Beijing Institute of New Technology, Beijing, China) provided a variable voltage of 0–30 kV across the capillary, with the outlet of the capillary at ground potential. A 60 cm fused-silica capillary (25 μm I.D., 375 μm O.D.) from Yongnian Optical Conductive Fiber Plant (Yongnian, China) was placed between two buffer reservoirs. A high voltage was applied at the injection end, while the reservoir containing the ECD cell was held at ground potential. Separations were carried out at an applied voltage of 20 kV. The ECD at a constant potential was performed with a voltammetric analyzer (Model CHI 800; Shanghai Chenhua Instrument Company, Shanghai, China). The detection cell was housed in a Faraday cage in order to minimize the interference from external sources of noise. ECD was carried out with a three-electrode system consisted of a carbon fiber micro-disk bundle electrode as the working electrode, a saturated calomel electrode (SCE) as the reference electrode, and a coiled Pt wire (0.5 mm diameter, 4 cm in length) placed at the bottom of the cell as the auxiliary electrode. The Pt wire was also served as the ground for the high-potential drop across the capillary.

2.2. Reagents and solutions

Trypsin (1:250) (tissue culture grade, from porcine pancreas, >250.N.F.U/mg) was obtained from Solarbio (Beijing, China). D-Hanks stock solution consisted of 1.4 mol/l NaCl, 0.0017 mol/l Na_2HPO_4•$12H_2O$, 0.054 mol/l KCl and 0.0044 mol/l KH_2PO_4. D-Hanks working solution was prepared by diluting the D-Hanks stock solution 10 times with deionized water. Sodium dodecylsulfate (SDS) was purchased from Sigma (St. Louis, Mo., USA). Dopamine (DA) and epinephrine (E) were obtained from Fluka (Buchs, Switzerland). Tyrosine was purchased from Xin Xing Medicine Health Products Technological Development Centers (Shanghai, China). Tryptophan (Trp) was purchased from Dong Fang Sanitary Materials Factory (Tianjin, China). AA (analytical grade, content >99.7%) was purchased from BASF Chemicals Limited Company (BASF, Ludwigshafen, Germany). All chemicals and reagents were analytical grade and used without further purification. All solutions were prepared with deionized water. Stock buffer solutions were 0.02 mol/l Na_2HPO_4 and 0.02 mol/l NaH_2PO_4. 0.01 mol/l stock solutions of AA were freshly prepared with water daily because of its sensitivity to air. The detected analytes were diluted with buffer solutions before analysis. The above solutions were stored at 4 °C in a refrigerator. In experiments, all solutions were sonicated for 3 min to degas just before use. Adult male rats (150–200 g) were purchased from Qingdao Pore Medicine Inspection Institute (Qingdao, China).

2.3. Fabrication of carbon fiber micro-disk bundle working electrode

A device for fabrication of CFMBE was shown in Fig. 1. About 30–40 carbon fibers (*ca.* 6 μm diameter, 7 cm in length) soaking up acetone were carefully inserted into a fused-silica capillary (*ca.* 250 μm I.D., 375 μm O.D., 1.5 cm in length) (Fig. 1A). Then the fused-silica capillary with the carbon fiber array was inserted and fixed into a steel tube (400 μm I.D., 700 μm O.D., 4 cm in length) by using ethyl α-cyanoacrylate adhesive. A copper wire's tip (400 μm diameter, 4 cm in length) sharpened by polishing on the abrasive paper was inserted into the other end of the steel tube and well adjusted

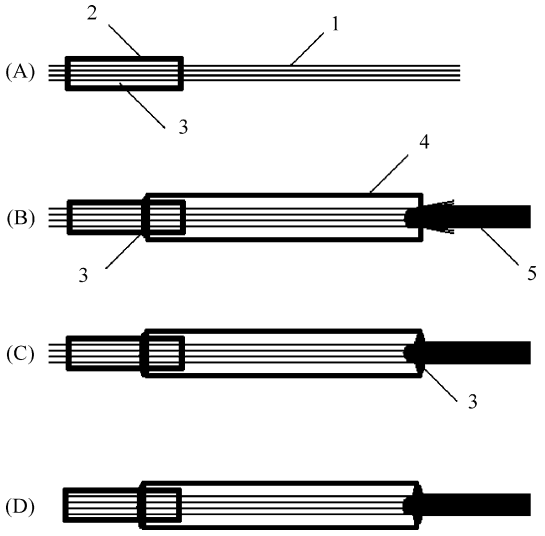

Fig. 1. (A–D) The schematic diagram of the CFMBE fabrication procedure. 1, Carbon fiber; 2, fused-silica capillary; 3, ethyl α-cyanoacrylate adhesive; 4, steel tube; 5, copper wire.

to connected with the carbon fiber array (Fig. 1B). The copper wire was also bonded to the steel tube with ethyl α-cyanoacrylate adhesive (Fig. 1C). The carbon fibers around the copper wire outside the steel tube were burned off by alcohol burner. Finally, cut the carbon fiber array protruding from the fused-silica capillary (Fig. 1D). Prior to CE analysis, all electrodes were cleaned in alcohol and washed with double distilled water for 5 min by using a supersonic wave cleaner.

Compared with the CFMBEs we previously used, the new kind of CFMBE used in this study was more easily fabricated because in the old style CFMBE, a very fragile glass capillary was used to contain mercury, while in the new kind of CFMBE, a hard steel tube was used instead of the brittle glass capillary. This made the fabrication easy. Further more, the new kind of CFMBE was more friendly to the environment because of free mercury. And the small size of the new CFMBE made it flexible and convenient to be used in the narrow detection cell.

2.4. Preparation of rat hepatocytes

A wide variety of methods have been employed for the isolation of rat hepatocytes [19,28,29]. In order to minimize the influence on the AA concentration either in the cell's extract or in the single cells by the preparation method, we isolated the rat hepatocytes with the enzymic-digestion method at the low temperature of 4 °C for 48 h in the icebox. Compared with the other hepatocytes isolation methods, this low-temperature enzymic-digest method could get high-yield and high-viability rat hepatocytes. Adult rat hepatocytes were isolated as follows. An adult male rat was killed by cervical dislocation, and the liver lobes were removed and put into 75% ethanol solution for about 2–3 min to be sterilized. After transferred into a 100-ml aseptical beaker and flushed twice with 10 ml D-Hanks working solution by pipet to remove excess blood, the liver lobes were cut into slices of 0.5–1.0 mm thickness and washed with 10 ml D-Hanks working solution twice again. These slices were then placed in a 250-ml conical flask containing 6-fold 0.25% trypsin solution and put in the icebox for 48 h at 4 °C for digestion. The resulting cloudy tissue suspension was obtained after the digestion and filtered through a layer of nylon mesh (150 μm pore, Zhejiang Shangyu Hujiang Instrument Mesh Factory, Shangyu, China) so as to remove large cell clumps and undigested material. The filtrate was

centrifuged at approximately 800 rpm for 10 min, and the pellet was washed three times in 5 ml D-Hanks working solution. The rat hepatocytes suspension was obtained by resuspending the cell pellet in 4 ml D-Hanks working solution by employing pipetting and stored under the temperature of 4 °C for further use. This solution was used to calculate the cell yield using a hemocytometer (Zhejiang Yuhuan Medical Instrument Plant, Zhejiang, China) and the amount was 9.76×10^5 cells/ml. About 1 ml of rat hepatocytes suspension was centrifuged at 800 rpm for 10 min and the supernatant liquid was removed. Then the rat hepatocytes were resuspended in running buffer (1.83×10^{-2} mol/l Na_2HPO_4–1.70×10^{-3} mol/l NaH_2PO_4 (pH 7.8)) with the same volume. After sonicated for 20 min to lyse, the cell solution was centrifuged for 10 min at 4000 rpm. The supernatant was the rat hepatocytes extract.

2.5. Procedure of single-cell analysis

The rat hepatocytes suspension was placed on a clear microscope slide and the slide was put on the inverted microscope (Nanjing Jiangnan Optical Instrument Limited Company, Nanjing, China) with a magnification of 160×. The injection end of the capillary filled with electrophoresis buffer was gently immersed in the hepatocytes suspension diluted under the guidance of a three-dimensional micromanipulator. In order to see the opening of the injection end, a *ca.* 5 mm of the polyimide-coating capillary was removed by burning. A platinum wire was placed in the cell suspension to serve as the electrophoresis anode. When a hepatocyte was drifting toward the injection end under the field of microscope vision, an injection voltage of 2 kV was applied to transport the whole cell into the capillary tip. Then, the injection voltage of 2 kV was turned off and the hepatocytes suspension on the microscope slide was replaced by 0.1% SDS to lyse. An injection voltage of 2 kV was applied for 5 s to inject SDS into the capillary tip. Lysis of the hepatocyte was observed with the aid of the microscope.

The capillaries were flushed with 0.2 mol/l NaOH, double distilled water, and the corresponding separation electrolyte for *ca.* 2 min, respectively, by means of a syringe before each run. After the electroosmotic current reached a constant value, the electromigration injection and lysis of the whole cell mentioned above was carried out. Then the capillary was put carefully to the CE electrophoresis buffer. Finally, a separation voltage of 20.0 kV was applied across the capillary and the detection potential of 0.80 V *vs.* SCE was applied at the working electrode and the electropherogram was recorded.

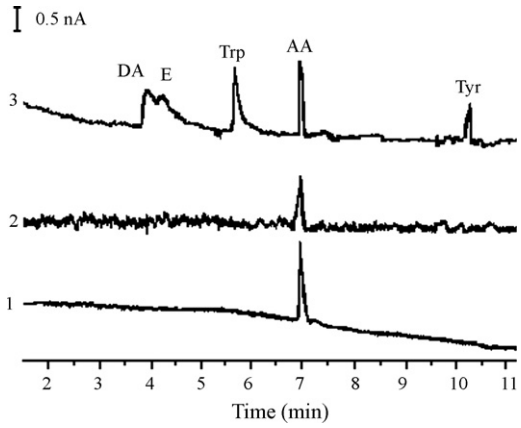

Fig. 2. Electropherograms of (1) 5.0×10^{-5} mol/l AA; (2) the extract of rat hepatocytes; (3) a solution containing 8.0×10^{-5} mol/l dopamine (DA), epinephrine (E), tryptophan (Trp), tyrosine (Tyr) and 5.0×10^{-5} mol/l AA. Running buffer, 1.83×10^{-2} mol/l Na_2HPO_4–1.70×10^{-3} mol/l NaH_2PO_4 (pH 7.8); capillary, 25 μm I.D., 375 μm O.D., 60 cm in length; separation voltage, 20.0 kV; injection, 5.0 kV for 10 s; detection potential, 0.80 V (*vs.* SCE).

2.6. Data treatment

Both AA in rat hepatocytes extract and in individual rat hepatocytes were identified by comparing their migration times with the migration times of standard AA in the electropherograms. Peak currents and the concentration standard calibration method were used for AA quantitation in rat hepatocytes extract. Since the size of the individual rat hepatocytes varied from 25–30 μm in diameter and their volumes were only approximately 8.2–14 pl per cell, thus there were different dilution factors in the determination of AA in cell extract and in the single cells. So we used peak areas and mass external standardization method for AA quantitation in single cells to ignore the differences due to the dilution factors.

3. Results and discussion

3.1. Detection of standard AA

We investigated the detection conditions for standard AA at a new kind of carbon fiber micro-disk bundle electrode with ECD. Under the optimum conditions of detecting AA by CE: 1.83×10^{-2} mol/l Na_2HPO_4–1.70×10^{-3} mol/l NaH_2PO_4 (pH 7.8) for running buffer, 20.0 kV for separation voltage, 0.80 V for detection potential, the electropherogram of 5.0×10^{-5} mol/l AA is shown in curve 1 (Fig. 2). The peak eluting at 7.02 min can be identified as the peak of AA, with a width at the peak half-height, $W_{1/2}$, of 4 s and theoretical plates, N, of 61370. The concentration detection limit (LOD_c) of AA was 1.7×10^{-6} mol/l, when S/N was 3, which is close to the calculated LOD_c 1.0×10^{-6} mol/l of AA by CE–ECD at a common CFMBE in Jin's paper in which the mass LOD of 168 amol and the injection volume of 0.168 nl [14], and is also close to the LOD_c 1.35×10^{-6} mol/l of AA by CE–ECD at a platinum electrode reported in Yao's paper [30].

Using the calculated injection volume of 1.75 nl, the mass LOD was 3.0 fmol. The concentration linear dynamic range was from 5.0×10^{-6} to 5.0×10^{-4} mol/l with the peak current regressive equation y nA = −5.137 nA + 0.838 (nA l/mol) × (mol/l) and a correlation coefficient of 0.9962 for the injection voltage of 5.0 kV and injection time of 10 s. The R.S.D. of the method for ten consecutive injections of 5.0×10^{-5} mol/l AA (n = 10) were 0.85% for the migration time and 1.8% for the peak current, respectively.

3.2. Determination of AA in hepatocyte extract

The electropherogram of the rat hepatocyte extract is shown in curve 2 (Fig. 2). It can be found that a peak, eluting at 7.02 min can be identified as corresponding to AA in the cell's extract on the basis of the migration time, as compared with the electropherogram of 5.0×10^{-5} mol/l AA shown in curve 1 (Fig. 2). In order to determine whether the other electroactive compounds, such as dopamine, epinephrine, tryptophan and tyrosine, which can be oxidized at the working electrode directly, interfered with the determination of AA, their electrophoretic behavior was investigated, and the electropherogram is shown in curve 3 (Fig. 2).

It was found that the peaks of these compounds could be well separated from AA, so they did not interfere with the determination of AA. The concentration of AA in the rat hepatocyte extract obtained by the standard calibration method is 3.6×10^{-5} mol/l. Fig. 3 shows the electropherograms of the rat hepatocyte extract without and with the defined added amount of AA. The recovery of the method was found to vary between 91 and 97%. Based on the cell

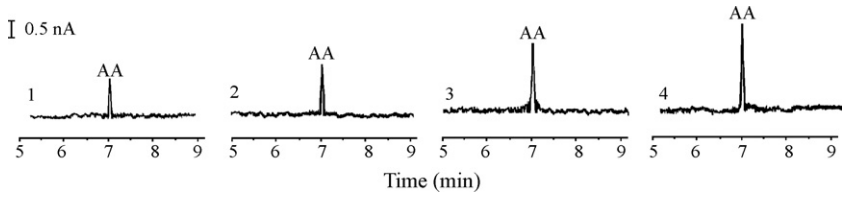

Fig. 3. Electropherograms of rat hepatocyte extract without and with the standard solution of AA. The added concentration of AA (mol/l): (1) 0; (2) 1.0×10^{-5}; (3) 3.0×10^{-5}; (4) 5.0×10^{-5}. Other conditions as in Fig. 2.

concentration in the rat hepatocyte extract of 9.76×10^5 cells/ml, the mean mass of AA in a single rat hepatocyte is calculated to be 37 fmol/cell.

3.3. Identification and quantification of AA in an individual rat hepatocyte

A typical electropherogram of the contents of an individual rat hepatocyte is shown in curve 3 (Fig. 4) appearing two peaks. Comparing curve 3 with curve 2 of the electropherogram of the D-Hanks working solution and curve 1 of the electropherogram of 5.0×10^{-5} mol/l AA, it can be found that the peak eluting at 2.21 min comes from D-Hanks working solution and the peak eluting at 7.02 min should be corresponding to AA based on the migration time. Fig. 5 depicts the electropherograms of three rat hepatocytes. In the experiment of the single-cell analysis for whole cell injection, the migration time of AA was almost the same with increasing the run number, but the peak widths of AA were not exactly the same probably because of the adsorption of the substances in the cells on the surface of the capillary wall although the capillary was flushed with NaOH solution, double distilled water and the running buffer before each run. So peak areas and mass external standardization method were used for the quantitative of AA in a single cell to minimize the calculate error brought by the broaden peak. The mass linear dynamic range was from 3.0 to 300 fmol with the peak area regressive equation y nC = 2.114 nC + 0.788 (nC/mol) × (fmol) and a correlation coefficient of 0.9962. The results of analysis for seven single hepatocytes are listed in Table 1, which shows that amounts of AA in single hepatocytes differ from cell to cell. The amount of AA in seven single rat hepatocytes ranged from 28 to 63 fmol, which is close to the amount of AA ranging from 16–62 fmol in single rat hepatocytes determined by capillary electrophoresis-electrochemiluminescence detection [31]. The value is higher than that in the hepatocytes extract 37 fmol/cell. If much more individual hepatocytes were analyzed, the values could be closer to each other.

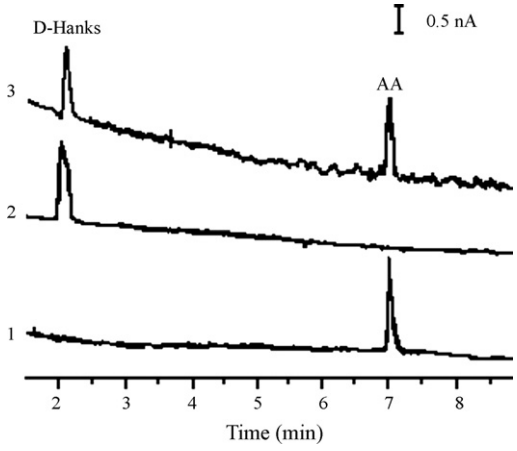

Fig. 4. Electropherograms of (1) 5.0×10^{-5} mol/l AA (2) D-Hanks working solution (3) a single rat hepatocyte. Conditions as in Fig. 2.

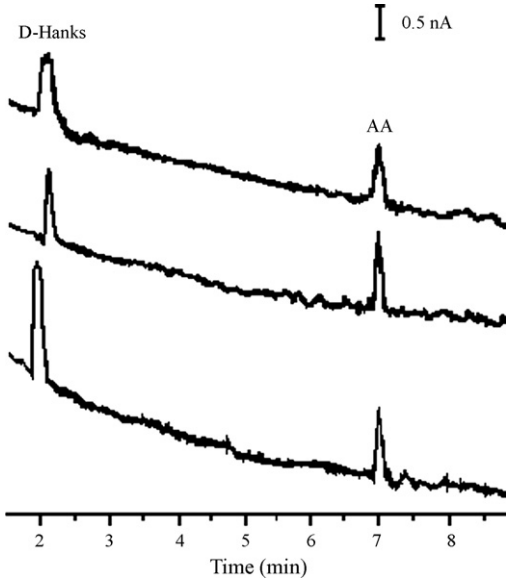

Fig. 5. Electropherograms of three individual rat hepatocytes. Conditions as in Fig. 2.

Table 1
Migration time, t_m, peak area, A, and amount of AA, q, in single hepatocytes

Run No.	t_m (min)	$10^9 A$ (C)	q (fmol)
1	7.08	3.31	36
2	6.95	5.15	63
3	7.07	2.72	28
4	6.98	4.56	54
5	7.02	3.95	45
6	7.05	3.31	36
7	7.02	4.49	53

4. Conclusions

In the present work, a new type of homemade CFMBE applied to CE–ECD has been developed for determination of AA in individual rat hepatocytes. Compared with the CFMBE we used previously, the new one is environmentally friendly, simple in fabrication procedure, and stable in the detection. This method has been successfully applied to the determination of AA in individual rat hepatocytes. It is proposed that this technique could be developed into a useful and powerful way for the analysis of chemical species in single cells.

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References

[1] B. Frei, L. England, B.N. Ames, Proc. Natl. Acad. Sci. U.S.A. 86 (1989) 6377.
[2] Mukaddes Eşrefoğlu, Mehmet Gül, Burhan Ateş, Kadir Batçıoğlu, Mukadder Ayşe Selimoğlu, World J. Gastroenterol. 12 (2006) 259.
[3] J. Lykkesfeldt, S. Loft, H.E. Poulsen, Anal. Biochem. 229 (1995) 329.
[4] J. Lykkesfeldt, H. Prieme, S. Loft, H.E. Poulsen, Brit. Med. J. 313 (1996) 91.
[5] J. Lykkesfeldt, S. Loft, J.B. Nielsen, H.E. Poulsen, Am. J. Clin. Nutr. 65 (1997) 959.
[6] M. De la Fuente, V.M. Victor, Free Radic. Res. 35 (2001) 73.
[7] J.Y. Kim, S.M. Lee, Life Sci. 75 (2004) 2015.
[8] P.H. Tan, P. Sagoo, C. Chan, J.B. Yates, J. Campbell, S.C. Beutelspacher, B.M. Foxwell, G. Lombardi, A.J. George, J. Immunol. 174 (2005) 7633.
[9] S. Harakeh, R. Jariwalla, AIDS Res. Hum. Retroviruses 13 (1997) 235.
[10] J.A. Jankowski, S. Trach, J.V. Sweedler, Trends Anal. Chem. 14 (1995) 170.
[11] E.S. Yeung, J. Chromatogr. A 830 (1999) 243.
[12] X.M. Sun, W.R. Jin, D. Li, Z.L. Bai, Electrophoresis 25 (2004) 1860.
[13] N. Gao, W.L. Wang, X.L. Zhang, W.R. Jin, X.F. Yin, Z.L. Fang, Anal. Chem. 78 (2006) 3213.
[14] W.R. Jin, L. Jiang, Electrophoresis 23 (2002) 2471.
[15] Q.F. Weng, W.R. Jin, Electrophoresis 22 (2001) 2797.
[16] X.M. Sun, W.R. Jin, Anal. Chem. 75 (2003) 6050.
[17] J.F. Liu, W.D. Cao, H.B. Qiu, X.H. Sun, X.R. Yang, E.K. Wang, Clin. Chem. 48 (2002) 1049.
[18] E.X. Vrouwe, R. Luttge, I. Vermes, A. van den Berg, Clin. Chem. 53 (2007) 117.
[19] W.R. Jin, X.J. Li, N. Gao, Anal. Chem. 75 (2003) 3859.
[20] W.R. Jin, W. Li, Q. Xu, Electrophoresis 21 (2000) 774.
[21] M. Pumera, X. Llopis, A. Merkoçi, S. Alegret, Microchim. Acta 152 (2006) 187.
[22] T.S. Zhou, F. Wu, G.Y. Shi, A.F. Wang, Q. Hu, X.C. Wang, Y.Z. Fang, Electrophoresis 27 (2006) 1790.
[23] W.R. Jin, D. Yu, Q. Dong, X.J. Ye, Electrophoresis 21 (2000) 925.
[24] W.R. Jin, Q. Dong, Electrophoresis 22 (2001) 2786.
[25] L.A. Woods, P.R. Powell, T.L. Paxon, A.G. Ewing, Electroanalysis 17 (2005) 1192.
[26] W. Wang, X.M. Sun, W.R. Jin, J. Chromatogr. B. 798 (2003) 175.
[27] W.R. Jin, Q.F. Weng, J. Wu, Anal. Chim. Acta 342 (1997) 67.
[28] J.R. Fry, C.A. Jones, P. Wiebkin, P. Bellemann, J.W. Bridges, Anal. Biochem. 71 (1976) 341.
[29] P.O. Seglen, Methods Cell Biol. 13 (1976) 29.
[30] X. Yao, Y.T. Wang, G. Chen, Biomed. Chromatogr. 21 (2007) 520.
[31] X.M. Sun, Y. Niu, S. Bi, S.S. Zhang, Electrophoresis, 2008, doi:10.1002/elps.200700792.