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Development of fully automated quantitative capillary electrophoresis with high accuracy and repeatability

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ABSTRACT: A quantitative capillary electrophoresis (qCE) was developed by utilizing a rotary type of nano-volume injector, an autosampler, and a thermostat with cooling capacity. The accuracy and precision were greatly improved compared with conventional capillary electrophoresis. The 10 nL volume accuracy was guaranteed by the carefully designed nano-injector with an accurate internal loop. The system repeatability (precision) in terms of RSD <0.5% for migration time and 1% for peak area were achieved by using DMSO as a test sample. We believe that this fully automated qCE system has the potential to be employed broadly in quality control and quality assurance in the pharmaceutical industry. Copyright © 2015 John Wiley & Sons, Ltd.

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Keywords: automation; electric decoupler; nano-valve; quantitative capillary electrophoresis

Introduction

In 1981, Jorgenson and Lukacs (1981) developed the first capillary electrophoresis system (CE), which was regarded as another significant progress in the advance of contemporary separation technology after high-performance liquid chromatography (HPLC). From the mid-to late 1980s, CE began to develop rapidly. As a powerful analytical separation technique characterized by high efficiency, high resolution and low consumption of solvent and sample, CE has established itself as a widely utilized technique for separation and analysis in many fields including environmental, pharmaceutical, biotechnology and food safety (Wang and Yan, 2012; Zhu *et al*., 2012; Simo *et al*., 2003; Rabanes *et al*., 2012; Yan, 2013). However, in practice, CE is still much less popular than HPLC, especially in quantitative analysis, such as pharmaceutical quality assurance and quality control (QC/QA). The major factor for the reluctant adaptation of CE is probably attributable to its 'dip-in' sample injection method, which has inherent defects, consequently resulting in poor accuracy and low precision (Tsuda *et al*., 1987; Fujiwara and Honda, 1986). Conventional injection in CE mainly includes electrokinetic injection and hydrodynamic injection, and both have disadvantages. Compared with the injection method in HPLC with a six-way valve, the accurate injection volume in CE is usually difficult to control, meaning that the amount of sample injected in CE is basically unknown. The lack of a real injection valve in CE also leads to poor repeatability (i.e. low precision) in quantitative analyses (Boone *et al*., 2002; Smith *et al*., 1991). In addition, the discrimination effects from electrokinetic injection can be a problem for sample mixtures with different charges (Feng *et al*., 2007). Problems in accuracy and precision are probably the major reason why CE remains less popular in its scope of utilization than HPLC (Zeng *et al*., 2012; Ye *et al*., 2011; Wang *et al*., 2012; Giordano *et al*., 2012).

Therefore, an injection method that can be utilized to reproducibly inject an accurate amount of sample into the CE system is critically needed. Several groups have made great efforts at developing a nano-injector with the purpose of improving the situation. Tsuda *et al*. (1987) initially introduced a nano-injector for CE in 1987 and succeeded in achieving an accurate injection amount of 350 nL. However, the injection volume was so large for CE with a capillary inner diameter lower than 100 μm that the resolutions of the peaks were seriously reduced. After Tsuda's work, Hanai and Tsuruta (1994) designed a slide-type injector, in which sample size was only 2 nL and the rotor was made from ruby. Although it was a major breakthrough in the progress of the valve injection method in CE, the dead volume in the valve junction was too large to obtain high resolution, which limited application of the slide-type nano-injector. In 2003, Iizuka *et al*. (2003) improved the rotary-type injector using a piece of fused-silica capillary that served as a sample chamber of 6–24 nL. In spite of the highly accurate and reliable quantitation, it was hard for the system to reach large-scale production because it was difficult to manufacture the precision valve. Our laboratory has been working on developing a controllable, repeatable, and accurate injection method in CE by adopting a four-way valve with a 4 or 10 nL internal loop. In 2003, Yao *et al*. (2003) established a quantitative

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Abbreviations used: BGE, back-ground electrolyte; CA, cellulose acetate; CE, capillary electrophoresis; DMSO, dimethylsulfoxide; EOF, electroosmotic flow; QA, quality assurance; qCE, quantitative capillary electrophoresis.

capillary electrophoresis system in which an injector of 10 nL was employed. With the rotary-type injector, an accurate volume injection and improved the repeatability were realized. However, the injection valve was placed in the electric field, which could cause electric shocks and bubble generation at high voltage when switching the valve between the 'load' and 'inject' positions. To overcome the limitations of the system, Li *et al*. (2009) constructed a new apparatus in 2009, in which an electric decoupler was designed and introduced into the new system with the aim of putting the injection valve outside the electric field so that the bubble generation and electric shocks could be avoided. However, with the nano-valve of 20 nL at the time, the sample volume was too large with respect to the inner diameter of 50 μm capillary (a sample plug of ~10 mm formed in the capillary) and it led to significant peak broadening. In addition, personal operation factors also contributed negatively to the repeatability with the manually operated system.

Herein, we report our development in a fully automatic quantitative capillary electrophoresis (qCE) system that consists of a laboratory-made autosampler equipped with a 10 nL rotary type injector and a laboratory-made thermostat with cooling capacity. The accuracy and precision were greatly improved compared with conventional capillary electrophoresis (CE). The 10 nL volume accuracy was guaranteed by the carefully designed nano-injector with an accurate internal loop. The system repeatability in terms of RSD $<0.5\%$ for migration time and $<1\%$ for peak area was achieved using DMSO as a test sample. We believe that this fully automated qCE system has a great potential to broaden the scope of practical applications of CE, especially in pharmaceutical QC/QA.

Materials and methods

Reagents and materials

All chemicals used were of analytical reagent grade unless indicated otherwise. Cytosine, 5-fluoro-2′-deoxyuridine, adenosine, uracil, uridine and creatinine were purchased from Shanghai Bio Science & Technology (Shanghai, China). Cellulose acetate (CA), methanol, sodium tetraborate, hydrochloric acid and sodium hydroxide were purchased from Sinopharm Chemical Reagent (Shanghai, China). All aqueous solutions were prepared with deionized water purified by a Mill-Q apparatus (Millipore, Bedford, MA, USA). All of the background solutions and samples were filtered using a 0.22 μm nylon filter (MSI, Westboro, MA, USA) and degassed by sonication. Fused-silica capillaries (50 and 100 μm i.d., 360 μm o.d.) were purchased from Polymicro Technologies (Phoenix, AZ, USA).

Automated qCE system

This apparatus consisted of a laboratory-made autosampler equipped with a four-way injector with a 10 nL sample loop (C4N-4344-.01/13R-0131H, Valco Inc., Houston, TX, USA), a syringe pump (LSP02-1A, Baoding LongerPump, Hebei, China) and a laboratory-made display panel. The high-voltage power supply, the variable-wavelength UV–vis absorbance detector and the chromatography workstation (TriSep-2003 chromatography) were all from Unimicro Technologies, Pleasanton, CA, USA. An electrical decoupler was fabricated in-laboratory and used to isolate the injector from the electrical field. A thermostat with cooling capacity was also constructed to remove the Joule heat and minimize the temperature fluctuation in the separation capillary.

Experiment and sample preparation

Prior to use, the new capillary was flushed with methanol, 0.1 mol/L HCl, purified water and 0.1 mol/L NaOH for 5 min, respectively, and finally with back-ground electrolyte (BGE) for 10 min. The capillary-flushing, which was accomplished automatically in the system, had profound effects on the stability of the electroosmotic flow (EOF) and the repeatability of the migration time. Seven nucleosides were weighed and dissolved with purified water to prepare stock solutions with a concentration of 1 mg/mL. These standard solutions were stored at 4°C in the refrigerator. They were diluted to the desired concentration with BGE when performing the experiment. All solutions were filtered through a 0.22 μm membrane filter (MSI, Westboro, MA, USA) and degassed by sonication (Benchtop Cleaners HS3120) before use.

Results and discussion

Automation

All of the modules of the quantitative CE system were automatically controlled by a computer program. The diagram of the apparatus is illustrated in Fig. 1. A six-port distribution and a three-port syringe pump were joined by a 30 cm long loop made from 400 μm i.d. PEEK tubing in the autosampler. The major functions of the autosampler were implemented by the six-port distribution and the three-port syringe pump and the loop between them in such a way that the process of capillary-flush, sample introduction and probe flushing could be fully automated.

When the injector is in the state of 'load' position, the internal loop connected the 'S' and 'W' ports. The sample was pumped into the loop by the three-port syringe pump. After switching the path in the six-port distribution, samples were injected into the internal loop by positive pressure. Then the injector was switched to the state of 'inject' position while the internal loop connected 'C' and 'P' ports in line with the separation capillary. Subsequently, the syringe pump filled with buffer drove the sample into the separation capillary. Afterwards, the injector was switched back to the 'load' state and the internal loop connected 'S' and 'W' again, ready for the next automatic flushing, injection and separation cycle. The utilization of the autosampler greatly improved the repeatability of migration time as well as peak area because errors and inconsistency from personal operation factors were avoided.

The preparation of electrical decoupler

It is a challenge to find the proper way to apply high voltage across the separation capillary in such a qCE system since the capillary inlet is connected to the nano-injector. We tried to apply the voltage in front of the injector by inserting the electrode into the splitter without success, probably owing to the complexity of the injector internal structure. Therefore, we had to find a way to isolate the injector from the electric field, that is, to apply voltage after (i.e. down-stream) the injector (see Fig. 1). We tried many ways, such as using a micro-Tee connector in which an electrode was connected to one port and the other two ports were used to connect the separation capillary. However, all methods failed for the reason that the bubbles resulting from electrode reaction could not be discharged from the connector and the bubbles finally found a way into the capillary, leading to electric current breakage.

Eventually, an electrical 'decoupler' was made in-laboratory and used to isolate the injector from the electrical field. Firstly, a

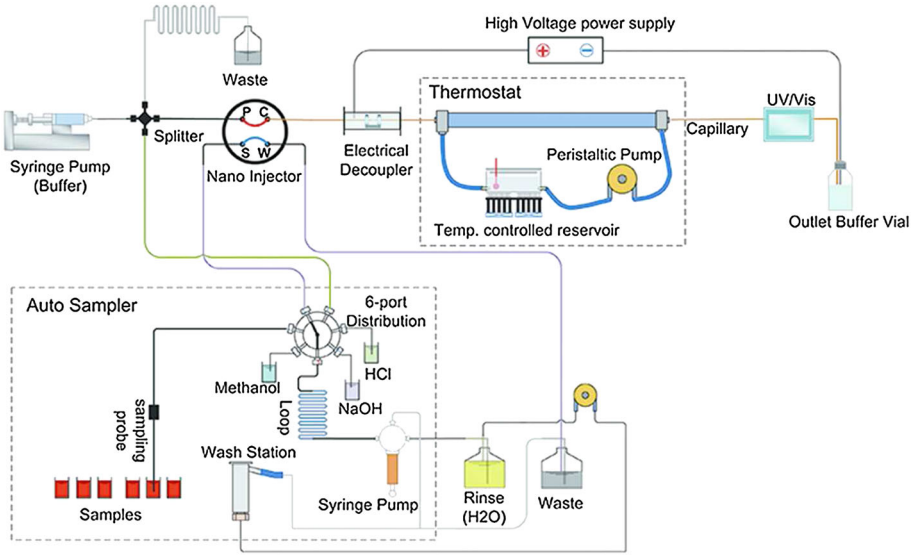

Figure 1. Schematic overview of automated quantitative capillary electrophoresis system.

U-shape plastic holder was employed to fix a capillary (50 μm i.d., 360 μm o.d., desired length) with AB rubber (a frequently used glue) tightly, as shown in Fig. 2(a). Secondly, at about 5 cm to the inlet end of the capillary, a fracture was made on the wall of the capillary within the holder. Then a few drops of 10% cellulose acetate solution (in acetone) were carefully dripped onto the fracture. After the acetone had evaporated, a layer of cellulose acetate membrane was formed around the fracture. Finally, the U-shape plastic holder was placed in a plastic columnar buffer reservoir in which the ground electrode was inserted, as shown in Fig. 2(b). This membrane allowed electrical current to flow between the buffer in the 2 mL decoupler and the capillary but without permeating the buffer and sample to pass through the membrane.

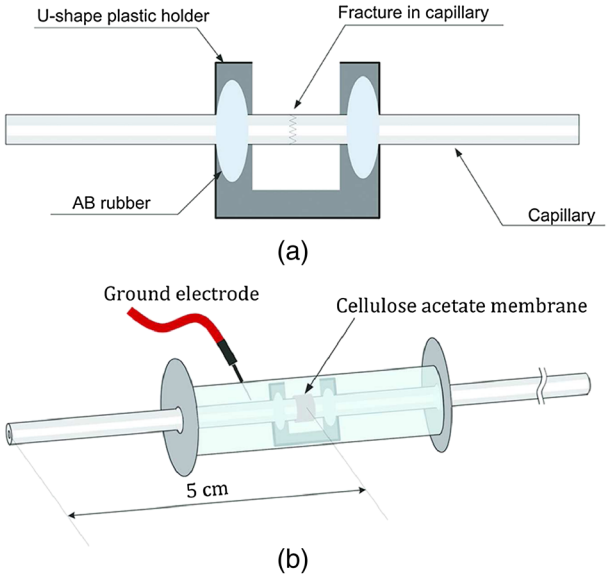

Figure 2. (a) Schematic overview of U-shape plastic holder with capillary; (b) schematic overview of electrical decoupler.

Repeatability of the device with the thermostat

It is well known that Joule heat in CE is unavoidable and it has negative impact on separation repeatability and reliability. Therefore, we designed and constructed a thermostat with cooling capacity. An experiment was designed to investigate systematically the effects of temperature on repeatability of migration time and peak area. As shown in Table 1, the repeatability of migration time and peak area were all significantly improved when the qCE experiments were performed at low temperatures. Especially compared with that in room temperature, the RSD of peak area reduced to below 1% and the repeatability for inter-day in 4 days also improved at low temperatures. The thermostat was indeed favorable for the performance of the qCE system.

Separation efficiency and resolution

The separation efficiency in our qCE system was somewhat lower than that in pure CE. Firstly, there was a small dead volume in the nano-injector in the port connecting the internal loop and

Table 1. The RSD intra- and inter-day for 4 days at different temperatures

	The RSD of intra-day								The RSD of inter-day	
	Day 1		Day 2		Da		Day 4		Four days	
	RSD of time (%)	RSD of peak area (%)	RSD of time (%)	RSD of peak area (%)	RSD of time (%)	RSD of peak area (%)	RSD of time (%)	RSD of peak area (%)	RSD of time (%)	RSD of peak area (%)
25 °C	0.99	1.58	0.94	1.72	0.49	1.31	0.64	1.98	2.27%	3.15%
12 °C	0.48	0.62	0.41	0.54	0.48	0.69	0.17	0.59	1.48%	1.74%

capillary inlet, which caused peak broadening when the 10 nL sample passed through it. Secondly, the profile of the EOF was plug-like and the pressurized flow was parabolic (Kanoatov *et al*., 2012; Jong and Sergey, 2012). In this qCE system, a gentle pressurized flow provided by a syringe pump (upper left in Fig. 1) was necessary to supply the BGE (a few hundred nanoliters per minute flow rate) for the separation capillary. The parabolic pressurized flow was superimposed on EOF, which led to a lower separation efficiency compared with that in traditional 'pure' CE, in which the flow profile was 'pure plug' like. In addition, a small fraction of separation capillary (about 5 cm, see Fig. 2b) between the electrical decoupler and the injector was not under the electric field. The BGE and sample were driven only by pressurized flow when they went through this section, which inevitably caused a parabolic flow, consequently leading to lower separation efficiency.

To determine the losses of separation efficiency, comparative experiments were carried out with both the qCE and traditional CE under the same voltage. A mixture of three negatively charged nucleosides, 5-fluoro-2′-deoxyuridine, adenosine and uridine, was chosen as test sample for this investigation. As shown in Fig. 3, the migration times of three nucleosides in the CE system were apparently longer than that in qCE, in which a gentle pressurized flow was superimposed on the EOF. The separation efficiencies of these three nucleosides in the qCE system were, respectively, 195.282, 142.877 and 158.182 plates/m, and the loss ratios of plates were respectively 27, 39.6 and 31.9% compared with that in traditional CE. However, the resolution in this case is obviously more than adequate.

Quantitative analysis of six nucleosides

To confirm the practical application of the automated qCE system, we employed it in the analysis of the six nucleosides, namely, cytosine, 5-fluoro-2′-deoxyuridine, adenosine, uracil, uridine and creatinine. The concentrations of all six nucleosides were 5.6×10^{-5} g/mL. Five nucleosides are all negatively charged except for cytosine, which is neutral. The separation conditions of the six nucleosides were optimized. Figure 4 illustrates the influence of the applied voltage on the resolution and migration time of six nucleosides when the syringe pump was set at 1.0 μL/min constant flow rate. As expected, the migration time for each compound became shorter gradually with the increasing voltage. A value of 15 kV was chosen as the optimum voltage in consideration of the resolution and migration time of the six nucleosides. The effect of the flow rate on the resolution and migration time of these nucleosides was also investigated and the electropherograms are shown in Fig. 5. Obviously, the migration time decreased gradually with the increasing flow rate of the syringe pump. The separation efficiency increased with decreasing flow rate of the syringe pump. This makes sense because in general the overall flow profile produced by both EOF and pressurized flow in qCE is somewhat parabolic, which is not in favor of separation efficiency. Thus, theoretically, the lower the pressurized flow is, the better the efficiency is. However, in practice, the efficiency started to decrease when the pressurized flow was too low. The reason for this phenomenon may be that, at certain point, the pressurized flow is not adequate for the EOF, resulting in a 'reversed parabolic' flow profile, which would damage the separation efficiency.

The repeatability of migration time and peak area of the six nucleosides was investigated before and after using the thermostat, as shown in Table 2 (the concentrations of all six nucleosides were 5.6×10^{-5} g/mL). The RSDs of cytosine were 0.64% for migration time and 1.50% for peak area without thermostat. However, the RSDs of cytosine were improved to 0.51% for migration time and 0.50% for peak area at 12°C with the thermostat. Apparently, charged species have a worse repeatability than neutral ones, probably owing to the electrostatic interaction with the capillary surface.

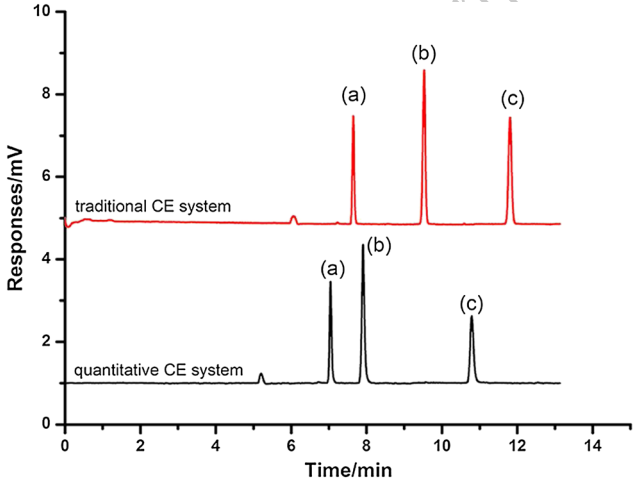

Figure 3. The comparison of separation of three nucleosides with the traditional capillary electrophoresis (CE) system (upper part) and quantitative capillary electrophoresis (qCE) system (the lower part). Capillary, 40 cm effective length, 50 μm i.d., 360 μm o.d.; voltage, 15 kV; temperature, 12°C; wavelength, 254 nm; injection volume, 10 nL; buffer, 30 mM sodium borate (pH 9.26); pressurized flow by syringe pump, 1.0 μL/min; split ratio, 13:1 (no electric field). Samples: (a) 5-fluoro-2′-deoxyuridine, (b) adenosine and (c) uridine.

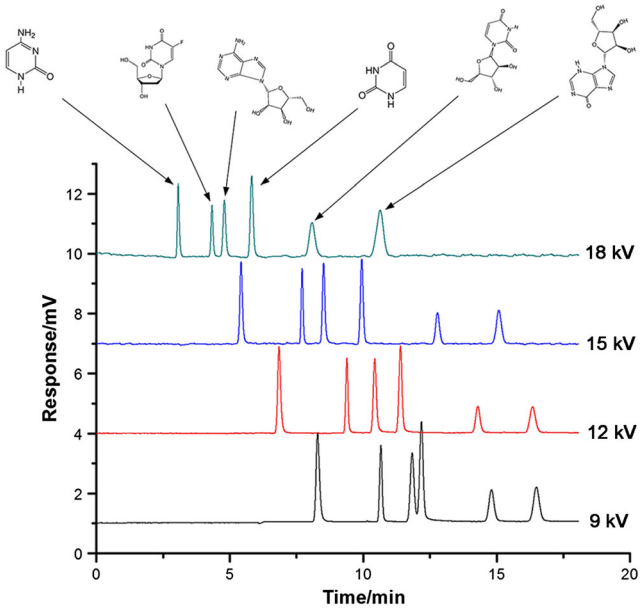

Figure 4. The effect of applied voltages on the resolution and migration time of nucleosides at constant pump flow rate (1.0 μL/min). Conditions are the same as in Fig. 3. Samples in sequence: cytosine, 5-fluoro-2′-deoxyuridine, adenosine, uracil, uridine and creatinine.

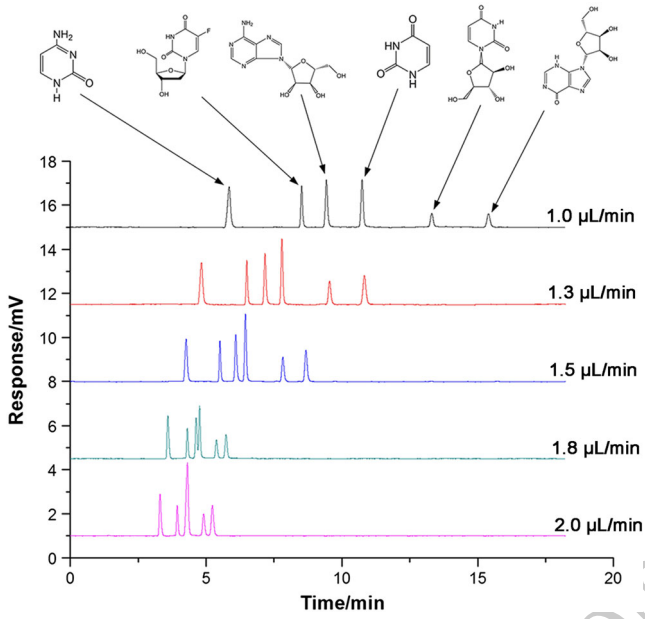

Figure 5. The effect of different flow rates of the syringe pump on the resolution and migration times of nucleosides at constant voltage (15 kV). Conditions are the same as in Fig. 3. Samples are the same as in Fig. 4.

Table 2. Repeatability of six nucleosides before and after using thermostat

	No thermostat		With thermostat (at 12°C)	
	RSD of time	RSD of peak area	RSD of time	RSD of peak area
Cytosine	0.64%	1.50%	0.51%	0.50%
5- Fluoro-2′-deoxyuridine	1.11%	1.98%	0.56%	0.83%
Adenosine	1.00%	1.92%	0.58%	1.56%
Uracil	1.88%	2.82%	0.50%	2.39%
Uridine	2.00%	2.84%	0.52%	2.46%
Creatinine	1.96%	4.55%	0.48%	2.66%

Conclusions

A fully automated quantitative capillary electrophoresis system was developed and characterized with high precision and accuracy. The repeatability in terms of relative standard deviation (RSD) of 0.50% for peak area and 0.51% for migration time was achieved with the thermostat when cytosine was used as the test sample. The feasibility of the apparatus was demonstrated by the baseline separation of six nucleosides.

Although there exist some sacrifices in the separation efficiency in this qCE system, it still holds considerable promise to be applied in quantitative analyses, such as pharmaceutical QC/QA. To extend the utility of this system further, we are exploring the development of other types of detectors such as laser-induced fluorescence detectors, evaporative light scattering detectors and electrochemical detectors. Work in these directions is in progress.

Acknowledgments

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References

Boone CM, Manetto G, Tagliaro F, Waterval JCM, Underberg WJM, Franke JP, de Zeeuw RA and Ensing K. Interlaboratory reproducibility of mobility parameters in capillary electrophoresis for substance identification in systematic toxicological analysis. *Electrophoresis* 2002; **23**: 67–73.

Feng YL, Lian HZ and Zhu JP. Application of pressure assisted electrokinetic injection technique in the measurements of DNA oligonucleotides and their adducts using capillary electrophoresis–mass spectrometry. *Journal of Chromatography, A* 2007; **1148**: 244–249.

Fujiwara S and Honda S. Determination of cinnamic acid and its analogs by electrophoresis in a fused silica capillary tube. *Analytical Chemistry* 1986; **58**: 1811–1814.

Giordano BC, Burgi DS, Hart SJ and Terray A. On-line sample preconcentration in microfluidic devices: a review. *Analytica Chimica Acta* 2012; **718**: 11–24.

Hanai T and Tsuruta H. Two-nanolitre injector for capillary zone electrophoresis. *Instrument Science and Technology* 1994; **22**: 151–155.

Iizuka E, Tsuda T, Munesue M and Samizo S. Nanoinjector for capillary electrophoresis and capillary electrochromatography. *Analytical Chemistry* 2003; **75**: 3929–3933.

Jong SD and Sergey NK. Pressure-based approach for the analysis of protein adsorption in capillary electrophoresis. *Analytical Chemistry* 2012; **84**: 453–458.

Jorgenson JW and Lukacs KD. Zone electrophoresis in open-tubular glass capillaries. *Analytical Chemistry* 1981; **53**: 1298–1302.
Li MJ, Zhou JY, Gu X, Wang Y, Huang XJ and Yan C. Quantitative capillary electrophoresis and its application in analysis of alkaloids in tea, coffee, coca cola, and theophylline tablets. *Journal of Separation Science* 2009; **32**: 267–274.
Kanoatov M, Retif C, Cherney LT and Krylov SN. Peak-shape correction to symmetry for pressure-driven sample injection in capillary electrophoresis. *Analytical Chemistry* 2012; **84**: 149–154.
Rabanes RH, Guidote AM and Quirino JP. Capillary electrophoresis of natural products: Highlights of the last five years (2006–2010). *Electrophoresis* 2012; **33**: 180–195.
Simo C, Barbas C and Cifuentes A. Chiral electromigration methods in food analysis. *Electrophoresis* 2003; **24**: 2431–2441.
Smith SC, Strasters JK and Khaledi MG. Influence of operating parameters on reproducibility in capillary electrophoresis. *Journal of Chromatography, A* 1991; **559**: 57–68.
Tsuda T, Mizuno T and Akiyama J. Rotary-type injector for capillary zone electrophoresis. *Analytical Chemistry* 1987; **59**: 799–800.
Wang MM and Yan XP. Fabrication of graphene oxide nanosheets incorporated monolithic column via one-step room temperature polymerization for capillary electrochromatography. *Analytical Chemistry* 2012; **84**: 39–44.
Wang ZF, Cheng S, Ge SL, Wang H, Wang QJ, He PG and Fang YZ. Ultrasensitive detection of bacteria by microchip electrophoresis based on multiple-concentration approaches combining chitosan sweeping, field-amplified sample stacking, and reversed-field stacking. *Analytical Chemistry* 2012; **84**: 1687–1694.
Yan C. *Contemporary Microscale Separation Technology*. HNB: New York, 2013.
Yao CY, Gao RY and Yan C. Quantitative sample injection for capillary electrophoresis. *Journal of Separation Science* 2003; **26**: 37–42.
Ye HZ, Xia SF, Yu LS, Xu XQ, Zheng CS, Xu HF, Wang LL, Liu XX, Cai ZW and Chen GN. Solid-phase extraction-field-amplified sample injection coupled with CE-ESI-MS for online pre-concentration and quantitative analysis of brain-gut peptides. *Electrophoresis* 2011; **32**: 2823–2829.
Zeng HL, Weng Y, Ikeda S, Nakagawa Y, Nakajima H and Uchiyama K. Accurate and highly reproducible picoliter injection system for capillary electrophoresis. *Analytical Chemistry* 2012; **84**: 10537–10542.
Zhu YW, Zhou CR, Qin SS, Ren ZY, Zhang LY, Fu HG and Zhang WB. A novel open-tubular capillary electrochromatography with magnetic nanoparticle coating as stationary phase. *Electrophoresis* 2012; **33**: 340–347.

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