

Chuanyi Yao<sup>a)</sup>,  
Ruyu Gao<sup>b)</sup>,  
Chao Yan<sup>a,c)</sup>

## Quantitative sample injection for capillary electrophoresis

<sup>a)</sup> Unimicro Technologies, Inc.,  
4713 First Street, Pleasanton,  
CA 94566, USA

<sup>b)</sup> State Key Laboratory, Institute  
of Element-Organic Chemistry,  
Nan Kai University, Tian Jin  
300071, China

<sup>c)</sup> Department of Biotechnology,  
Dalian Institute of Chemical  
Physics, Chinese Academy of  
Science, 161 Zhongshan Road,  
Dalian 116011, China

An apparatus including a rotary-type injector was designed for quantitative sample injection in capillary electrophoresis (CE), in which both pressurized flow and electroosmotic flow were used to drive the background electrolyte solution. A relative standard deviation of peak area of lower than 1% was achieved by using this apparatus. The effects of back-pressure regulator, restrictor, and applied voltage on separation efficiency and resolution were investigated. The utility of this apparatus in both micro-HPLC and pressurized capillary electrochromatography (pCEC) was also demonstrated.

**Key Words:** Quantitative sample injection; Capillary electrophoresis; Capillary electrochromatography; Pressurized CEC; Micro-HPLC

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### 1 Introduction

Capillary electrophoresis (CE) has become a relatively mature technique in separations of complex mixtures due to its high efficiency, high speed, and small sample size. However, for the past 20 years, commercially available CE instruments have suffered from a major weakness, which is the lack of a reliable and quantitative injection method. Consequently, CE has limited applications in quality control and quality assurance, where regulatory compliance demands accuracy, precision, and reliability.

Sample introduction in CE can be accomplished in a number of ways. The electrokinetic method and the hydrodynamic method are most commonly used [1]. In the electrokinetic method, the inlet end of a capillary and an electrode are placed in a sample reservoir and a voltage is briefly applied. The solutes in the sample migrate into the capillary at speeds ( $u_{\text{sam}}$ ) dependent on the electroosmosis ( $u_{\text{EOF}}$ ) and their electrophoretic mobilities ( $u_{\text{mob}}$ ).

$$u_{\text{sam}} = u_{\text{EOF}} + u_{\text{mob}} \quad (1)$$

The sample amount injected into the capillary is generally controlled by the applied electric field and applied time. However, many parameters of sample solution such as pH, viscosity, ionic strength, dielectric constant, and temperature affect electroosmosis and electrophoretic mobility. Although the electrokinetic method is generally reliable and fully adopted by commercial CE instruments, it suffers from discrimination of the solutes within the sam-

ple. Because the electrophoretic mobility ( $u_{\text{mob}}$ ) of each solute is dependent on the number of charges on the solute and its molecular size, the amount of a solute injected depends on its nature. In an extreme case, when the electrophoretic migration direction of a solute is against the electroosmotic flow (EOF) and  $|u_{\text{mob}}|$  of the solute is larger than  $|u_{\text{EOF}}|$ , the solute will not migrate into the column.

In the hydrodynamic method, the sample is injected into the capillary by a pressure difference between the inlet and outlet ends. The pressure difference is generated by placing the capillary ends at different levels or by applying a constant pressure to the sample reservoir or a vacuum to the outlet end of the capillary. The amount of the sample injected into the capillary is controlled by the pressure difference and the time applied.

Both the electrokinetic and hydrodynamic methods require the following procedure to accomplish the injection: first, the inlet end of the capillary is removed from the electrolyte vial and dipped into the sample solution; secondly, an electric field or a pressure is applied to inject the sample; thirdly, the capillary is transferred back into the electrolyte vial. This "dip in" method has major problems of accuracy and precision. It is difficult to inject an accurate amount of sample by the "dip in" method because the amount of sample can only be estimated after an experiment by using the flow rate and applied time. In addition, the residual amount of sample (usually a droplet) outside of the inlet end of the capillary is affected by many parameters such as the viscosity, surface tension, and temperature. Therefore, it is very difficult to inject an accurate and precise amount of sample into the column. Moreover, the transfer steps during the injection procedure need to interrupt the applied voltage. After the separation begins, there

**Correspondence:** Chao Yan, Unimicro Technologies, Inc.,  
4713 First Street, Pleasanton, CA 94566, USA.  
Phone: +1 925 846 8638. Fax: +1 925 846 3687.  
E-mail: chaoyan@unimicrotech.com.

is always a period of time before the system reaches its stabilized condition. Therefore, there is a critical need for a sample injection method for CE that can be used to inject an accurate amount of sample reproducibly.

Zare and Tsuda [2] designed a capillary electrophoresis system comprising an interface device with a capillary and a tubing inserted therein. A sample is injected through the tubing into the interface and is thereafter introduced into the capillary column, in which the electrophoretic separation is performed. By employing the interface device, the injection method permits samples to be introduced into the capillary without the need to disengage or to alter the electric field. Virtanen [3] described a system in which the sample is injected by means of a sample-injection capillary placed in the vicinity of the inlet end of a separation capillary. In such a manner, the sample solution surrounds the inlet end entirely, and sample is transferred into the separation capillary by EOF and electrophoretic mobility or by other means. After a predetermined time the solution is withdrawn from the vicinity of the inlet end, whereupon the sample solution is replaced by the background solution. These two methods have the advantage of an injection without interruption of the applied voltage, but are not able to accurately control the amount of sample introduced.

Recknor et al. [4] introduced a pressure injection apparatus for CE. In their system, a servo pressure regulator is incorporated to generate a constant pressure in communication with sample reservoir for a predetermined period of time. The reproducibility of sample introduction was improved with the RSD of peak area lower than 2%. However, the accurate amount of sample injected by this method is still very difficult to estimate.

The rotary-type injector commonly used in liquid chromatography is most suitable for the repeated injection of an absolute amount of sample. However, it has not been used in CE for the following reasons. First, when an HPLC injection valve is used in CE without supplementary pressure, bubbles are usually generated inside the injector (as was found in our experiments), causing the electrical current to stop. Secondly, it is very difficult to fabricate and utilize an injector with extremely small volume (typically 10 nL) without introducing a significant dead volume. Tsuda [5] used an injector with the rotor and stator made of ceramic material for sample introduction in CE. However, the injection amount of 350 nL was too large for a capillary with an inside diameter (ID) lower than 100  $\mu\text{m}$ . In this article, we report our investigation on quantitative sample injection with a rotary-type injector of 10 nL volume for CE. A reproducibility of injection with RSD of peak area lower than 1% was achieved. The effects of applied pressure, back-pressure, applied voltage, and polarity on column efficiency and resolution were also

investigated. The rotary-type injector was coupled to a versatile instrument, which could be also used for pressurized capillary electrochromatography (pCEC) and micro-HPLC with solvent gradient capability.

## 2 Experimental

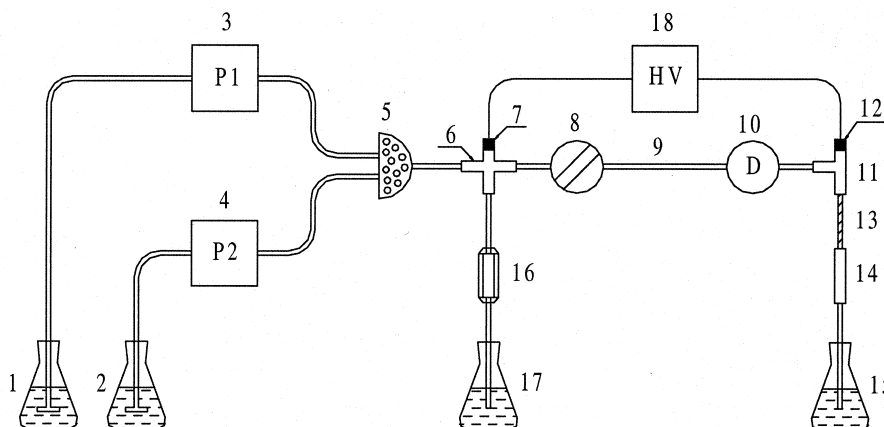
### 2.1 Materials

Fused silica capillaries were purchased from Polymicro Technologies Inc. (Phoenix, AZ, USA). Columns used as restrictor were supplied by Unimicro Technologies, Inc. (Pleasanton, CA, USA). Water was purified with a Milli-Q apparatus (Millipore, Bedford, MA, USA). Methanol of HPLC grade was purchased from Merck (Darmstadt, Germany). Sodium dihydrogen phosphate, disodium hydrogen phosphate, thiourea, *p*-methoxybenzoic acid, and *p*-benzenedicarboxylic acid (Beijing Chemicals Company, Beijing, China) were all of analytical grade. All the background solutions and samples were filtered by a 0.22  $\mu\text{m}$  nylon filter (MSI, Westboro, MA, USA) and degassed by sonication.

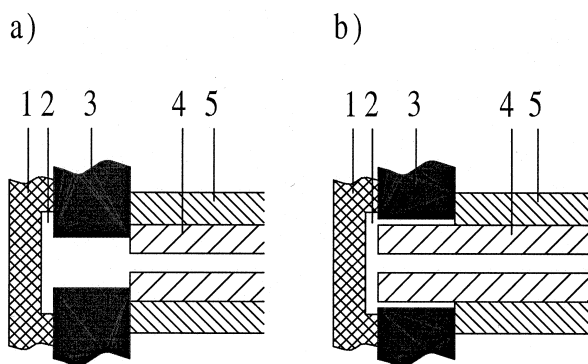
### 2.2 Instrumental

A TriSep™-2000GV system [6] (Unimicro Technologies, Inc., Pleasanton, CA, USA), on which pCEC and micro-HPLC can be performed, was modified as shown in **Figure 1** for the CE separation with quantitative sample introduction. The background solutions (1 and 2) are mixed at the mixer. Then the mixed solution flows to the splitting cross, where the grounded electrode is placed. Part of the solution flows through the injector and into the capillary column, which is connected with a Tee where another electrode is placed. Another part of the solution flows through a back-pressure regulator and to the waste reservoir. The back-pressure regulator and the flow restrictor (a 320  $\mu\text{m}$  ID column packed with 5  $\mu\text{m}$  spherical silica particles) connected with the Tee are designed to control the flow velocity in the capillary column and maintain a pressure on the solution in the capillary to avoid bubble formation. A variable-wavelength UV/Vis absorbance detector (HD-VUV-20) (Unimicro Technologies, Inc., Pleasanton, CA, USA) was used in all the experiments.

A commercially available 4-port nano-volume sample injector (C4-0004-.01) of 10 nL (Valco, Inc., Houston, TX, USA) was modified to reduce the dead volume. As seen from **Figure 2.a**, the diameter (360  $\mu\text{m}$ ) of the port in the injector is smaller than the outer diameter (375  $\mu\text{m}$ ) of a capillary. The capillary can not be inserted into the port and consequently there is a 78 nL dead volume. But for the through port injector (**Figure 2.b**), the diameter of the same port is enlarged to 390  $\mu\text{m}$ , so the capillary can be inserted into the port and the dead volume is significantly



**Figure 1.** Apparatus for quantitative sample injection in CE. 1. Background solution A, 2. Background solution B, 3. Pump 1, 4. Pump 2, 5. Mixer, 6. Splitting cross, 7. Ground, 8. Rotary-type injector, 9. Capillary column, 10. UV/Vis detector, 11. Tee, 12. Electrode, 13. Restrictor, 14. Union, 15. Waste reservoir, 16. Back-pressure regulator, 17. Waste reservoir, 18. High voltage power supply.



**Figure 2.** Schematic diagram of the injector port in connection with capillary column.

1. Rotor, 2. Sample loading slot, 3. Stator, 4. Capillary column, 5. PEEK tubing.

a) Normal injector. b) Through port injector.

reduced. All the injectors used in the experiments were manually operated with a lab-made handle of polymethyl methacrylate which replaced the metal handle. The internal flow paths are all metal-free to avoid unnecessary electrochemical reaction. In some experiments where the column efficiency was not a concern, the valve without modification was used. Although the instrument is capable of performing solvent gradient operation, only isocratic results are reported in this paper.

### 3 Results and discussion

#### 3.1 Set up of apparatus

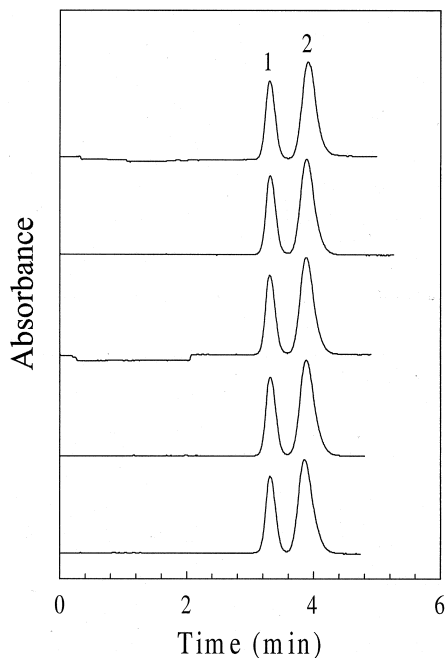
At the beginning, we tried to use the injector directly for CE without the pressurized flow and restrictor. However, the system was not stable with the electroosmosis-driven

flow because of bubbles generated inside the injector. The bubble formation may be due to the difference in surface properties between the flow path inside the injector and the capillary. Applying pressure to the background solution was considered to be effective in inhibiting bubble formation. The back-pressure regulator is used to control the pressure added to the background solution in the capillary column. The flow rate in the capillary column can be adjusted by selection of suitable restrictor columns with different lengths and internal diameters as well as the particle size of packing materials. In our experiments, it was found that the existence of pressurized flow made a major contribution to the inhibition of bubble formation. CE separation was successfully achieved with the apparatus shown in Figure 1.

When the open capillary for CE separation is replaced by a packed capillary column, both pressurized CEC and micro-HPLC can be performed by such an apparatus. In the pressurized CEC mode, the mobile phase is driven by pressurized flow and electroosmosis. The ratio of pressurized flow and electroosmotic flow can be adjusted by the change of back-pressure regulator, restrictor, and voltage, facilitating the fine-tuning of the selectivity of a separation for both charged and neutral components.

#### 3.2 Reproducibility

The purpose of designing such an apparatus is to investigate the feasibility of using an HPLC-type injector for CE while achieving a quantitative and precise injection. A sample mixture containing solutes of thiourea and *p*-methoxybenzoic acid was used to test the reproducibility. The results are shown in **Figure 3** and **Table 1**. The two solutes were successfully separated. Each time, the



**Figure 3.** Reproducibility of injection. Capillary: 100  $\mu\text{m}$  ID, overall length 40 cm, effective length 30 cm. Background solution: phosphate buffer (pH 7)/methanol = 80:20, 5 mM (after mixing). Pump flow rate: 0.01 mL/min. Voltage: 200 V/cm. Back-pressure: 250 psi. Restrictor: 320  $\mu\text{m}$  ID  $\times$  10 cm, packed with 3  $\mu\text{m}$  ODS. Detection: 254 nm. Sample: 1. thiourea, 2. *p*-methoxybenzoic acid.

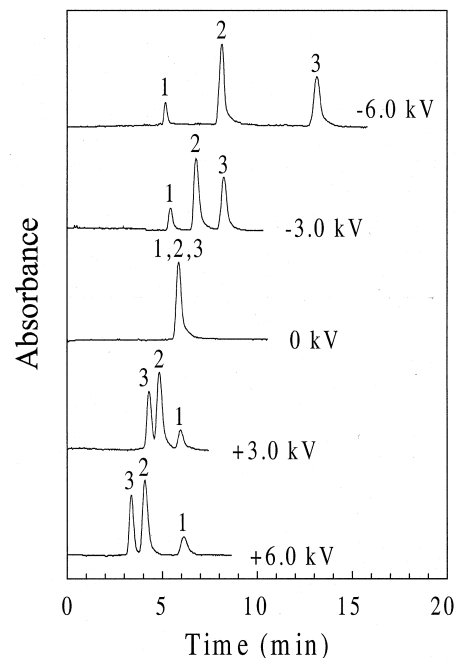
amount of 10 nL mixture was accurately injected and the experiment was repeated five times. The reproducibility of peak areas was significantly improved with a RSD of 0.95% for peak 1 and 0.84% for peak 2. As in HPLC, these results are quite acceptable in quantitative analysis.

### 3.3 Reversed electroosmosis direction

The electroosmotic flow is generally from the anode to the cathode in a silica capillary. Therefore, in a conventional CE system, the cathode is usually set at the outlet end and the anode is set at the inlet end of the capillary with the detector located near the outlet end. If the polarity of the electrode is switched, the sample may not be introduced. However, for the apparatus shown in Figure 1, the electroosmosis can be in a reversed direction because of the existence of pressurized flow. A solute migrates at speed ( $u_{\text{sol}}$ ) depending on the pressurized flow ( $u_{\text{pre}}$ ), electroosmotic flow ( $u_{\text{EOF}}$ ), and its electrophoretic mobility ( $u_{\text{mob}}$ ).

$$u_{\text{sol}} = u_{\text{pre}} + u_{\text{EOF}} + u_{\text{mob}} \quad (2)$$

When the splitting cross in Figure 1 is grounded and a positive voltage is applied to the Tee, the EOF is in the direction from the detector to the inlet end of the capillary. As long as  $|u_{\text{pre}} + u_{\text{EOF}} + u_{\text{mob}}| > 0$ , the overall flow is still from the inlet end of the capillary to the detector and the



**Figure 4.** CE electropherogram at different voltages. Capillary: 50  $\mu\text{m}$  ID, overall length 30 cm, effective length 20 cm. Background solution: phosphate buffer (pH 7)/methanol = 80:20, 5 mM (after mixing). Pump flow rate: 0.01 mL/min. Back-pressure: 40 psi. Restrictor: 320  $\mu\text{m}$  ID  $\times$  20 cm, packed with 5  $\mu\text{m}$  bare silica. Detection: 254 nm. Sample: 1. thiourea, 2. *p*-methoxybenzoic acid, 3. *p*-benzenedicarboxylic acid. Voltages shown on the electropherogram are the voltages applied to the Tee.

**Table 1.** Reproducibility of quantitative sample introduction in CE.

	Retention time ( $t_R$ ) [min]		Peak area [mVs]	
	peak 1	peak 2	peak 1	peak 2
Average value	2.787	3.268	5.857	10.471
RSD [%]	0.138	0.479	0.953	0.838

sample can be introduced. In our experiments, the positive or negative electrode was connected to the Tee (outlet) and the cross (inlet) was always grounded. The results are shown in Figure 4. Obviously, when the voltage was set to zero, the solutes flowed at the same speed and could not be separated. When the negative electrode was connected to the Tee, the neutral thiourea (peak 1 in Figure 4) would elute faster than the case of zero voltage because the EOF was superimposed upon the pressurized flow (Table 2). The electrophoretic forces made the negatively charged solutes flow slower than thiourea so the separation was achieved. The retention time of thiourea decreased and the resolution increased with the increase of voltage across the column. When a positive

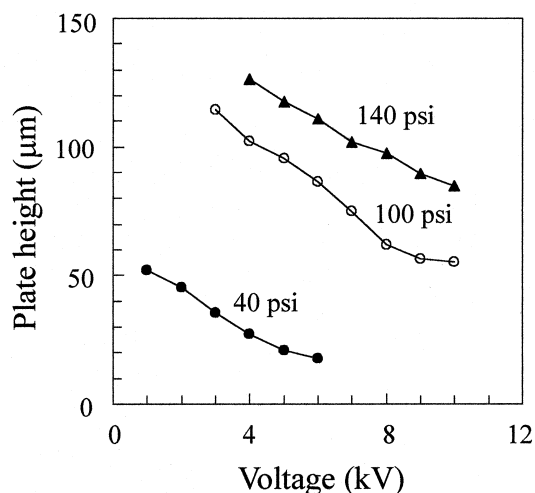
**Table 2.** Retention time and resolution at different voltage.

Voltage [kV]	$t_R$ [min] peak 1	peak 2	peak 3	Resolution of peak 1 and peak 2
6.0	5.406	3.590	2.956	1.69
3.0	5.286	4.290	3.803	1.17
0	5.187	5.187	5.187	0
-3.0	4.806	5.993	7.286	3.18
-6.0	4.560	7.186	11.603	8.01

electrode was connected to the Tee, the order of the peaks was reversed. In this case, the retention time of thiourea and the resolution were both increased with the increase of voltage. This kind of separation can not be successfully achieved with a conventional CE instrument because of the absence of pressurized flow.

### 3.4 Effects of back-pressure regulator and restrictor

In the CE system illustrated in Figure 1, the pressurized flow is controlled by the back-pressure regulator and the restrictor while the EOF is controlled by the voltage applied over the column. The pressurized flow has a parabolic flow profile, while the EOF has a plug flow profile. It is expected that the column efficiency increases with increasing contribution of EOF to the overall flow. As shown in **Figure 5**, the plate height indeed decreased with the increase of the voltage. The sample mixture of thiourea and *p*-methoxybenzoic acid was used. The negative electrode was connected to the Tee (see Figure 1). The peak of *p*-methoxybenzoic acid was selected to calculate the column efficiency. It was found that the resolution increased with increasing voltage. At a fixed voltage, the plate height increased and the resolution decreased



**Figure 5.** Effect of back-pressure on efficiency. Back-pressure: ● 40 psi, ○ 100 psi, ▲ 140 psi. Other conditions are the same as in Figure 4.

with increasing back-pressure. Therefore, from the standpoint of efficiency, it is favorable to decrease the back-pressure and increase the voltage. However, it should be noted that this would increase the possibility of bubble formation.

The experiments also showed that the column efficiency decreased with a shorter restrictor at a fixed voltage and a certain back-pressure regulator. The decrease of efficiency was caused by the increasing contribution of the pressurized flow.

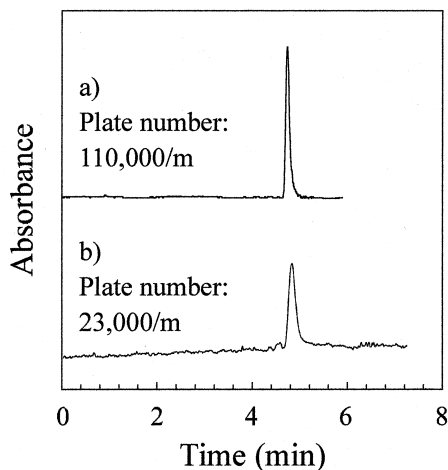
### 3.5 Through-port injector

The column efficiency in this CE system is lower than that obtained with a conventional CE system. Besides the parabolic flow profile caused by the pressurized flow, the dead volume in the injector is also one of the main reasons. As can be seen from Figure 2.a, there is a columnar cavity between the inlet end of the capillary and the sample slot in the injector. The cavity, which has a volume of about 78 nL, may be the main source of dead volume. A “through-port injector” as in Figure 2.b was designed. The diameter of the columnar cavity in the new injector is little bigger than the external diameter of the capillary. The capillary can get through the cavity to nearly touch the rotor. The dead volume is significantly decreased. When the new injector was utilized, the efficiency increased to a value of 110,000 plates/m (see **Figure 6**).

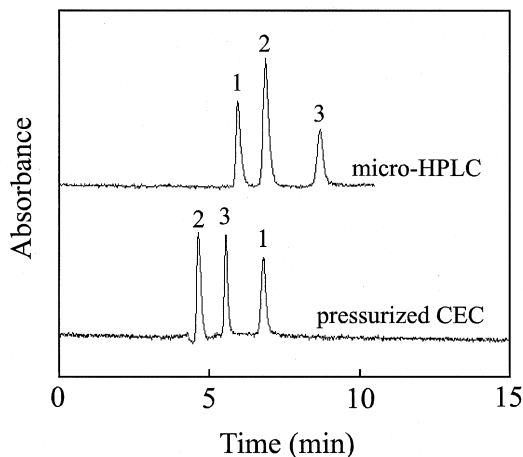
Although the column efficiency in the CE system described here is lower than in conventional CE due to the existence of pressurized flow and the dead volume in the injector, quantitative sample injection is successfully achieved. This brings bright prospects for CE applications in quantitative analysis such as in quality control and quality assurance in the pharmaceutical industry. Furthermore, the solvent gradient capability in CE may have great potential in certain applications.

### 3.6 Micro-HPLC and pressurized CEC

The apparatus shown in Figure 1 can also be used in the mode of micro-HPLC and pressurized CEC (pCEC) [7, 8]. When the open capillary is replaced with a packed column



**Figure 6.** Comparison of electropherograms obtained with through port injector (a) and unmodified injector (b). Voltage: 10 kV. Sample: thiourea. Other conditions are the same as in Figure 4.



**Figure 7.** Separation of thiourea, benzaldehyde, and *p*-hydroxybenzoic acid on an ODS column in the mode of micro-HPLC and pressurized CEC. Column: 150  $\mu$ m ID, overall length 30 cm, effective length 20 cm, packed with 3  $\mu$ m ODS. Mobile phase: acetonitrile/water = 70/30 (v/v). Pump flow rate: 0.01 mL/min. Back-pressure: 1000 psi. Detection: 254 nm. Voltage for pressurized CEC: 167 V/cm. Sample: 1. *p*-hydroxybenzoic acid, 2. thiourea, 3. benzaldehyde.

and only pressure is used, the separation will be in the micro-HPLC mode. It becomes pCEC when both pressure and voltage are applied. An example is shown in **Figure 7**. An octadecyl (C18) ODS column was used to separate a sample mixture of *p*-hydroxybenzoic acid (peak 1), thiourea (peak 2), and benzaldehyde (peak 3) on the apparatus in the micro-HPLC and pCEC mode (Figure 7). The retention time of a neutral molecule is shorter in

pCEC than in micro-HPLC due to the contribution of EOF. However, the negatively charged *p*-hydroxybenzoic acid migrates slower in pCEC because of its electrophoretic mobility, which is in the opposite direction to the EOF and pressurized flow. The column efficiency is much higher in pCEC than that in micro-HPLC (81,000 plates/m, and 42,000 plates/m, respectively, calculated with the peak of benzaldehyde) because of the plug-like flow profile of EOF.

#### 4 Concluding remarks

An apparatus was designed for quantitative sample introduction in CE. The rotary-type injector was used to introduce an accurate and precise amount of sample. The background electrolyte solution is driven by electroosmosis and pressurized flow. By using such an apparatus, the reproducibility of peak areas is significantly improved with a RSD of lower than 1%. The efficiency is enhanced by increasing the contribution of electroosmosis and decreasing the contribution of pressurized flow to the overall flow. Although there are still difficulties associated with separation efficiency, the successful achievement of quantitative injection holds considerable promise for CE applications in both quality control and quality assurance. In addition, we believe the solvent-gradient capability has great potential in certain bioanalyses.

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