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Study on a Novel Two-dimensional HPLC and Wide-bore Electrophoresis System

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By precisely fixing an inner cooling capillary to the axial center of a quartz tube, a new and better wide-bore electrophoresis (WBE) system was built and has shown great potential in load capacity and separation efficiency. Further, a novel two-dimensional hybrid of HPLC and WBE system (HPLC–WBE) was developed with an injection valve as the interface. High-resolution and rapid analysis speed were observed in both the WBE system and the 2D HPLC–WBE system. Further work is currently underway.

The rapid development of proteomics and genomics imposes challenges to the analytical community to come up with more and more efficient analytical technologies capable of handling complex biological samples resulting from the whole collection of a biologic cell, tissue, or even an organ. Quite often, the samples are so complicated that a single existing separation technology alone cannot provide sufficient resolution for the need. Therefore, it is necessary to combine two or more separation technologies together to provide sufficient analytical power in separation and analysis of the samples. Two-dimensional (2D) techniques increase the peak capacity as well as resolution compared with any one of them. So far, 2D electrophoresis (2DE) is the best in terms of its ability to resolve thousands of proteins.[1] However, due to the difficulty in automation, it yields to the 2D liquid chromatography (2D-LC) in recent years.[2,3]

Though not comparable with 2DE, 2D-LC does offer reasonable resolution and can implement automation. However, 2D-LC itself is not immune to some problems. One big issue is the validity of selection of two orthogonal phases. Ideally, one is reversed phase while the other is ionic, which is charge-based. In practice, most compounds strongly retained on one stationary phase have the tendency to be strongly retained on the other stationary phase as well due to many secondary interaction mechanisms. Thus, an ingenious tuning of the operating parameters is often necessary to gain true orthogonal separations, which is not always easy to achieve. A true orthogonal 2D system can easily be built if a hydrophobicity-based LC and a charge-based electrophoresis, such as capillary electrophoresis (CE), is linked together as they have totally different separation mechanisms.[4,5]

Such an entirely orthogonal 2D LC–CE system, if achieved practically, would have great potential for complex bioassays. However, it is very challenging to directly detect the diluted fractions from LC in CE by the conventional UV detector, and it is very difficult to construct such a 2D LC–CE system primarily due to the extremely small sample volume in CE (usually a few nL). Such a small volume makes this 2D LC–CE system difficult to gain widespread use because it is a true art to interface these two techniques together and the impact of the LC mobile phase to the CE separation is significant. Both HPLC valve-based and the so called "flow-gating" interfaces as well as their modifications have been used with certain success.[6–9] Although useful for some simple samples, given the technical difficulties involved, the true usefulness of the 2D LC–CE system remains unclear. A more efficient and practical 2D technology is desirable.

The wide-bore electrophoresis (WBE) technology developed in our group deals with separation in a quartz tube with an internal diameter larger than 1 mm, which significantly increases the sample-loading capability without the concern of the Joule heat.[10] This offers an alternative to build a new 2D system, i.e., a 2D LC–WBE system, where a reversed-phase HPLC and a WBE system are connected through an injection valve.

The WBE technology contains an internal capillary coaxially inserted into a quartz tube to carry away the Joule heat in situ in real time by passing through coolant during electrophoresis. Earlier, we found that the inner cooling capillary tends to be off the center along the quartz tube due to gravity. This deviation affects the injection and separation efficiency. Thus, it is necessary to reduce this deviation and keep the capillary at the axial center position as much as possible. For this purpose, a 17.0 cm quartz tube (i.d./o.d. 1000/2500 μm) was drilled to form three holes at 1.0, 3.0, and 16.0 cm from one end. A short capillary (i.d./o.d. 75/365 μm) was inserted perpendicularly into the hole at 3.0 mm from the end and was aligned with the inner surface of the quartz tube for injecting samples. Then, the inner cooling capillary (i.d. 530/690 μm) was inserted coaxially into the quartz tube. Two pieces of hollow fiber tubes (0.69/1.00 mm i.d./o.d., 0.60 mm length) were placed between the cooling capillary and the quartz tube with one at each end to fix the capillary at the center. All junctions were sealed with epoxy glue. Thus formed WBE system had an assurance that the largest distance between the inner surface of the quartz tube and the outer surface of the cooling capillary was no more than 155 μm. This distance is similar to that in most CE systems; therefore, it is expected that the improved WBE system would strengthen the heat elimination ability and deliver better separation results.

Figure 1 manifests the optimal separation conditions and the complete separation of six aromatic acids in the improved WBE system. The separation efficiency achieved for *p*-toluenesulfonic acid, *o*-iodobenzoic acid, benzoic acid, phenylacetic acid, cinnamic acid, and nicotinic acid was 67872, 69769, 69019, 76197, 82105, and 60337 theoretical plates per meter, respectively. While the inner cooling capillary was fixed randomly inside the wide-bore tube, under the same conditions, the best separation efficiencies of the above components were 36020, 36062, 36867, 39108, 51757, and 26345 in three different WBE systems, respectively. This indicates that the modification of the cooling capillary inside the quartz tube does improve the separation efficiency in the WBE system.

With the rapid development of proteomics, it is of great value to have a new powerful technology for the separation and

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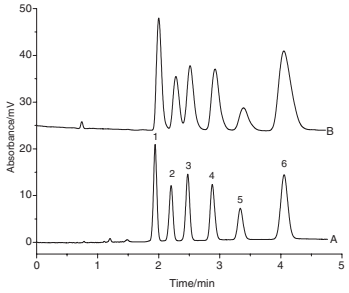

Figure 1. Electrophorograms of six acids in WBE with fixing precisely (A) and randomly (B) the position of the inner capillary. Experimental conditions: the total/effective separation length 15/8 cm, 3000 V, 20 mmol L^{-1} acetic acid at pH 4.0 containing 0.6% hydroxyethyl cellulose, detection at 214 nm, 15 mL min^{-1} coolant at 10 °C. 200 nL samples, (1) 0.01 mg mL^{-1} *p*-toluenesulfonic acid and 0.03 mg mL^{-1} (2) *o*-iodobenzoic acid, (3) benzoic acid, (4) phenylacetic acid, (5) cinnamic acid, and (6) nicotinic acid.

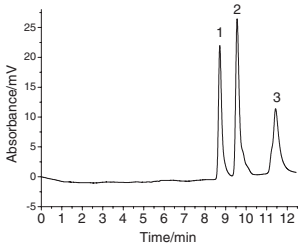

Figure 2. Electrophorogram of three basic proteins by WBE. Conditions: all conditions were the same as mentioned in Figure 1 except 0.1 mg mL^{-1} samples and 10 mmol L^{-1} phosphate buffer at pH 2.3, containing 0.6% hydroxyethyl cellulose. (1) lysozyme, (2) cytochrome *c*, (3) ribonuclease A.

analysis of proteins. CE has many advantages, such as shorter separation time, higher efficiency, and smaller volume requirement compared with traditional techniques for protein analysis, such as chromatography and slab gel electrophoresis. It is expected that WBE will offer similar potential for protein analysis as in CE while offering better sensitivity. However, WEB will suffer from the same problem of protein binding to uncoated capillaries in CE because they are fundamentally similar technologies. For this reason, a relatively low pH was chosen for evaluating the feasibility of using WBE for the analysis of proteins. Figure 2 shows the separation of three basic proteins in the improved WBE system. The results indicate that the WBE has high separation capability and is suitable for the analysis of both small and large molecules.

As we expected, the WBE system could offer a much larger volume of sample than a typical CE system. For example, the load capacity for a WBE system with 40-cm separation length was as high as 1.6 μL. Therefore, WBE system has the potential to be a semipreparative or even a preparative technique. At the same time, WBE offers the advantage of easier interface with other conventional technologies, such as HPLC, especially microbore HPLC, which has a similar internal diameter (1 mm) as the WBE. This has been demonstrated in our study of

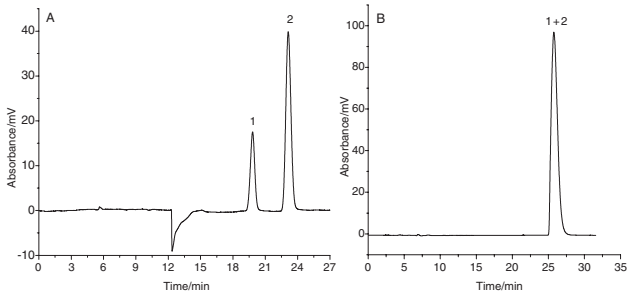

Figure 3. Separation of benzoic acid and phenylacetic acid on the HPLC-WBE system (A) and HPLC (B). Chromatographic conditions: 250 × 4.6 mm 5 μm, C_{18} LC column, the mobile phase, the mixture of methanol and pH 5.0 phosphate buffer (v/v 50:50), the flow rate, 0.8 mL min^{-1}, detection at 230 nm. WBE conditions: the total/effective length 32/40 cm, separation voltage: 6.0 kV; sample, 1000 nL the effluent from HPLC. Other conditions were the same as remained in Figure 1. (1) benzoic acid and (2) phenylacetic acid.

building a novel 2D-HPLC–WBE system. Differing from capillary LC–CE-based 2D systems commonly used in proteomics, our HPLC–WBE 2D system offers the advantages of more sample loading and easier connection with conventional systems while providing a true orthogonal 2D. To demonstrate the power of the HPLC–WBE system, two close compounds in structure, benzoic acid and phenylacetic acid, were used as the model for the evaluation of this 2D HPLC–WBE system in both off-line and on-line modes.

In off-line HPLC–WBE analysis, 20 μL 1.0 mg mL^{-1} sample mixture was loaded onto a C_{18} column and eluted simultaneously by the mobile phase. Fractions of the effluent from the HPLC system were separately collected into 0.5-mL vials, and were analyzed one by one in the WBE system. The electrophorogram in Figure 3A clearly shows the separation of these two compounds, which were not separated in the HPLC chromatogram alone as shown in Figure 3B.

Figure 3 indicates that the fractions collected from the HPLC can be directly analyzed by WBE without the need for any prior concentration. This is of great value as it may avoid the deterioration of proteins during the condensation, drying, and reconstitution process.

For on-line HPLC–WBE 2D analysis, a six-port injection valve was used to connect the HPLC and the WBE as shown in Figure 4. A capillary was used as the sample loop (400 nL). In order to make the 2D system work, it is necessary to shorten the analysis time of the second dimension. Therefore, the WBE channel was shortened to 15 cm. Figure 4 clearly shows that this system works well under the construction following these steps:

1) Loading the effluent from the LC column into the sample loop.

2) Injecting the effluent from the loop into the WBE system through pushing 1000 nL running buffer with a 10 μL syringe.

For example, Figure 5 is a WBE electrophorogram obtained from an on-line HPLC–WBE system for separating the HPLC effluent (Figure 3B) in the WBE system. It was clear that benzoic acid and phenylacetic acid, which were not separated by HPLC alone (Figure 3B), were very well separated in WBE.

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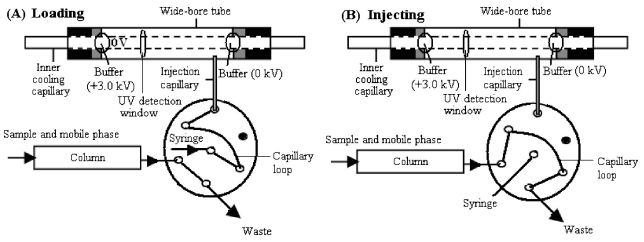

Figure 4. Schematic diagram of the interface between HPLC and WBE.

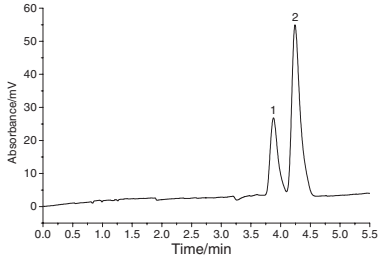

Figure 5. Electrophorogram of benzoic acid and phenylacetic acid in the HPLC–WBE system. Conditions: all conditions were the same as indicated in Figures 1 and 3 except electrophoresis samples. (1) benzoic acid and (2) phenylacetic acid.

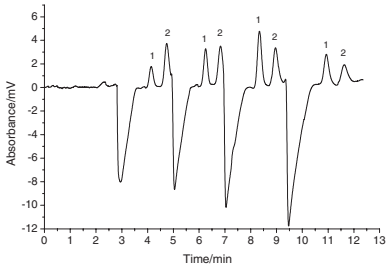

Figure 6. Electrophorogram of benzoic acid and phenylacetic acid in continuous HPLC–WBE analysis. Conditions: all conditions were the same as indicated in Figure 5 except the flow rate of the mobile phase, which was changed to 0.5 mL min^{-1}. (1) benzoic acid and (2) phenylacetic acid.

The name "stroboscopic" comes from a general principle of analyzing a rapid periodic process by consecutively sampling fractions from the same chromatographic peak in CE at least three/four times for on-line/off-line HPLC–CE analysis.[11] Similar methodology was adopted here for evaluating the potential in identifying hidden peak components. With a low flow rate, HPLC fractions were transferred into the WBE sequentially at ca. 2-min intervals. From the pattern as shown in Figure 6, it was concluded that the elution order of the two compounds was different in WBE vs. in HPLC; i.e., benzoic acid (marked 1) migrated out of the WBE first but was eluted from HPLC later as its peak size increased with time while phenylacetic acid did exactly the opposite.

To further shorten the time gap between two consecutive WBE injections, a novel idea of using multiple injection lines was adopted to divide the WBE channel into multiple sections. For example, three lines were positions on the separation channel for bringing components from HPLC into the WBE channel at three different times. With proper control of the timing, it is even possible to maximize the number of injections into the WBE channel.

In addition, the multiposition approach can also be used to study the length effect of the separation channel on separation efficiency simultaneously. It was found that the resolution rose from 1.688 to 2.569 when the separation channel was lengthened from 8 to 16 cm. As short as 60-s injection interval was achieved on this on-line HPLC–WBE system using a manual injection valve. It is expected that even shorter time is possible if an automated injection valve is used.

With precise positioning of the inner cooling capillary inside the quartz tube, a new WBE system was constructed and higher separation efficiency in small molecule analysis was obtained compared to an unimproved WBE system. Meanwhile, the complete separation of the proteins was accomplished successfully in the WBE system for the first time. Furthermore, the improved WBE system with 1.6 μL loading capacity provided a better basis for interfacing with HPLC. And the effluent from HPLC could be directly separated and detected in WBE without the need for any prior concentration. Moreover, the novel on-line 2D HPLC–WBE system was built and showed significant improvement in separation of two model compounds compared with HPLC alone. In addition, the interval between the WBE analysis was shortened from 120 to 60 s with the three injection line development, which could optimize simultaneously the effect of the channel length on separation. This powerful system has overcome several limitations in HPLC–CE and will offer much potential for broad applications. The analysis in protein degradation is currently underway.[12]

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