A comparison study of in-column and on-column detection for electrochromatography

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**A B S T R A C T**

Duplex capillary columns, the standard for electrochromatography using optical detection, consist of a packed and an open section. Normally, optical detection is performed in an on-column manner, i.e. at a point right after the packed section. It was deemed that band broadening may take place when an analyte band travels from the packed bed, through the frit and down to the open section. In this study, without using any sintering steps for fritting or window creation, robust packed capillary columns were prepared using transparent capillaries based on single particle fritting technology. The detection point could be easily shifted by simply sliding the transparent column against the ultraviolet (UV) beam. In this way, the band broadening effect was directly evaluated as a function of the detection point, which was positioned before or after the end frit. The consistent van Deemter curves recorded indicate that there was no efficiency difference between the positions investigated. The result proved that the significant band broadening effect previously observed via on-column detection should be caused by the sintered frit used, while the single particle frit made through a purely physical process did not lead to efficiency degradation. The conservative separation performance recorded at different positions around the column’s end also suggests the applicability of on-line tandem detection strategy, e.g. UV followed by mass spectrometry (MS), on the same capillary column, which should be a promising approach to mining multiple detection information from a single microseparation process.

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1. Introduction

Due to the combined chromatographic and electrophoretic mechanism [1], electrochromatography (EC) holds unique potential for bioanalysis at microscale, e.g., proteomics [2,3]. Apart from CEC–MS hyphenation, commonly used CEC columns have a duplex configuration [4,5], i.e., a packed section and an open section. The open section was left unpacked to enable on-column optical detection over it. In contrast, optical detection can also be directly performed over the packed bed (i.e., in-column detection) to eliminate the possible band broadening effect taking place in the open section. However, in-column detection may sacrifice sensitivity due to light scattering effects on the particulate packed bed [6–9]. Therefore, in electrochromatography, optical detection is usually performed right after the packed bed and over the open section. To this end, the commonly used polyimide coated capillaries need to be sintered/corroded to remove the non-transparent coating in order to create an optically transparent detection window. Most research groups have reported CEC separations using this on-column detection strategy [4,5]. With 3 μm ODS particles as an example, typical efficiencies in the range of 100,000–200,000 plates/meter have been reported [4,10]. Other CEC works performed with in-column detection have recorded higher plate numbers of 300,000–400,000 plates/meter using packing materials of the same size [7,11].

Recently, liquid chromatography hyphenated with tandem detection techniques is becoming popular due to the multidimensional and complementary detection information it can provide for comprehensive analysis of complex mixtures [12–14]. Such a strategy should also be applicable to microseparations. For example, CEC with in-column UV and on-column MS detection may be an ideal approach to acquiring both quantitative and qualitative...
information of the electroseparation [15]. This calls for capillary column technologies with good flexibility for the choice of detection strategies, as well as separation performance conservation for the subsequent combination or comparison of different detection outputs.

We have introduced a single particle fritting technology for electrochromatographic column fabrication [16–19]. Using this technology, without any sintering or chemical bonding processes, prefabricated frits can be lodged inside the capillary tube based on the keystone effect. In this study, using transparent capillaries and single particle fritting technology, we prepared robust capillary columns to directly evaluate band broadening effect through UV detection before and after the end frit. The primary aim was to clarify whether there is significant efficiency difference between in-column and on-column detection and what is the main cause for the difference.

2. Experimental

2.1. Materials and apparatus

Polymide coated fused silica capillaries, 100 μm i.d., 365 μm o.d. and 20 μm i.d., 90 μm o.d., were purchased from Yongnian Reafine Chromatography (Hebei, China). Transparent fused silica capillaries, 100 μm i.d., 365 μm o.d. were purchased from Composite Metal Services (Ilkley, UK). Perfluorosilica beads, 110 μm in diameter, pore size ~1 μm, to be used as frits of capillary columns, were obtained from X-tec (Bromborough, UK). Reversed phase packing material, Waters Spherisorb ODS1, 3 μm, from Waters (Milford, MA) was used for capillary column packing. Tris-(hydroxymethyl)aminomethane hydrochloride (Tris–HCl, biochemical grade), thiourea, methyl-, ethyl-, propyl- and butylbenzenes, acetone and acetonitrile (ACN, HPLC grade) were obtained from Sigma–Aldrich (St. Louis, MO). Ultrapure water (18.2 MΩ) was prepared in a Milli-Q system (Millipore Filter Co., Bedford, MA) and used throughout this study. All chemicals and reagents were at least of analytical or high grade and used without further purification. An Elite P230 high pressure pump from Dalian Elite Analytical Instruments (Dalian, China) was used for column packing. A Waters Quanta 4000E capillary electrophoresis system (Waters, Millford, MA) equipped with a single wavelength UV (214 nm) detector, without thermostating and pressurisation facilities, was used for electrochromatography separations.

2.2. Column preparation

The CEC columns used in this study were fabricated based on a single particle fritting method reported before [16]. In brief, a transparent capillary of ~32 cm long, 100 μm i.d. was chosen for packing. A single perfusive particle, ~110 μm in diameter, was tapped in from one end of the capillary. A narrow bore capillary, 90 μm o.d. was used as a plunger to push the particle inward to a desired position (8 cm in). This single particle was used as the outlet frit of the column. Then, from the other end of the transparent capillary, a slurry of the packing material was introduced and packed under a high pressure up to 6000 psi. When the column bed was packed to the length, another single particle was tapped in as the inlet frit. In this way, a duplex column (32 cm total length) with the long end (24 cm) packed and short end (8 cm) left open was prepared, as shown in Fig. 1. In the later stage of the study, another two transparent capillary columns of the same configuration and dimension were fabricated for efficiency consistency test.

2.3. Electrochromatographic separation and detection

The mobile phase was ACN/aqueous Tris–HCl (50 mM, pH 8.5), 80:20 v/v. The sample was a mixture of thiourea, methyl-, ethyl-, propyl- and butylbenzenes dissolved in the mobile phase, with each component having a concentration of ~0.5 mM. Both the mobile phase and sample solution were sonicated before use. All the samples were introduced electrokinetically at 5 kV for 5 s. The CEC experiments were performed at a room temperature of ~25 °C.

In terms of detection, five positions (as shown in Fig. 1) were chosen for in-column (A and B) and on-column (C, D and E) UV detection, respectively. Position A was 1 cm before the frit, positions B and C were right before and after the frit, and positions D and E were 1 and 2 cm after the frit, respectively.

3. Results and discussion

3.1. Multiple detection points on a single column

Since a transparent capillary was adopted and the single particle fritting technology does not need any sintering steps for frit fabrication, the capillary column was made in an absolutely non-destructive manner [16]. This is in stark contrast to sintered-fritted capillary columns, in which the capillary tube should be sintered at the frit and detection window area [20–23]. Care must be taken during column handling as the column is prone to break in the sintered area. While for the single particle fritted transparent capillary column, in addition to the intact tube strength, plenty of freedom was allowed for locating the optical detection point. As a matter of fact, one could perform UV detection at any desired positions along the column length, as shown in Fig. 1. This underpinned the proposed investigation, i.e., directly comparing in-column and on-column detection on the same column.

Using an alkylbenzene mixture as the sample, CEC separations were performed in the whole voltage range (1–30 kV) and detected at five different points as depicted in Fig. 1. The typical electrochromatograms recorded at 14, 16 and 20 kV are presented in Fig. 2.

In each frame of Fig. 2, the two electrochromatograms recorded via in-column detection (at points A and B, Fig. 1) show noisy baselines due to the light scattering effect on the particle-packed bed. Nevertheless, under the same applied voltage, different detection points did not record significantly different chromatogram pattern, apart from slight shifting to a longer run time from points A to E. Similarly, consistent chromatograms were recorded at the same detection point under different applied voltages, again with slight shifting due to the EOF change resulting from different applied voltages. It may be noticed that the peak height of methylbenzene was fluctuating between electrochromatograms. This is because the data series were taken from whole voltage range evaluations which took a long time to complete. During the course of the experiments, the sample was not frequently replenished and this led to decreased peak height of alkylbenzenes due to evaporation, especially for the short alkyl methylbenzene. Since the focus of the
present study was not on the quantitative aspect of the analytes, we only concentrated on the retention times and peak efficiencies of the alkylbenzenes.

3.2. Peak efficiency: in-column vs. on-column detection

The van Deemter curves obtained at the five detection points are shown in Fig. 3. The five curves have a highly similar pattern and all presented excellent efficiencies at the high speed range. With a retained species, butylbenzene, as the probe, plate heights of 6–7 μm were obtained at linear velocities from 0.5 to 2.7 mm/s (under applied voltages 8–30 kV). The present data indicates that, in-column (points A and B) and on-column (points C, D and E) detection made no difference to the separation efficiency: all recorded excellent peak efficiencies at ∼150,000 plates/meter.

The result of present study is in stark contrast to previous reports. Using polycyclic aromatic hydrocarbons (PAHs) as probes, Yan et al. obtained significantly improved peak efficiencies of up to 400,000 plates/meter via in-column detection [7]. Their columns were packed with a mixture of 3 μm SynChrom ODS particles (90%) and 1 μm silica gel (10%) through an electrokinetic packing method [24]. High peak efficiency based on in-column detection was also reported by Robson et al. [11]: up to 376,000 plates/meter were obtained for CEC of PAHs. The columns were packed with 3 μm Waters Spherisorb ODS1 particles through a supercritical fluid packing method. In contrast, Rebscher and Pyell [6] reported the in-column peak efficiency of bromobiphenyl at 37,000 plates on a 29.5 cm column (i.e. ∼120,000 plates/meter). Their column was packed with 3 μm Polygosil ODS particles through the slurry packing method, the same method used in the present work. These various observations are summarized in Table 1. Since all these studies utilized a nominal 3 μm ODS material in their in-column detection experiments, one factor contributing to the difference in the peak efficiencies may be the different packing strategies adopted. With electrokinetic and supercritical fluid packing methods, higher efficiencies were observed and this could be a sign of the better-packed bed (in terms of packing density and packed bed uniformity). The other parameter should be due to the size of the analyte. PAHs are larger molecules than alkylbenzenes, therefore have lower diffusion coefficients and narrower peak widths for a given time spent in the mobile phase.

The data from the present work have also shown that the peak width did not change when the analyte eluted through the frit, off the packed bed and down to the open section, on the single-particle fritted column. Such a conserved peak efficiency contrasts with the significantly changed efficiency observed via in-column (400,000 plates/meter) and on-column detection (110,000–150,000 plates/meter) on sinter-fritted columns packed with 3 μm ODS particles as Yan et al. reported [7]. Similar efficiency degradation effect was also observed by Dadoo et al. using sinter-fritted columns packed with 1.5 μm nonporous ODS material [25]. It has been well documented that sintered frits have a typical length of 2–6 mm [26], and sinter-fritting is not a well controlled process (temperature, sintering time etc.) [22,23]. This resulted in a relatively long frit whose surface charge state and flow path morphology were different from that of the packed bed [4,5]. Such sintered frits consequently led to the nonuniformity at the end of the packed bed [27–29]. Rebscher and Pyell [8] also pointed out that sinter-induced thermolysis of ODS material may give rise to adsorption of hydrophobic compounds on the carbonized surface of the frit, which resulted in strong peak tailing, i.e., efficiency degradation. These factors suggest that it was the very short length (∼100 μm) and the sinter-free nature of the single-particle frit that minimized the band broadening effect, and hence conserved the sharp band while the analyte passed through the frit. A direct comparison of a sintered frit and a single particle frit is provided in Fig. 4.
3.3. Estimation of band broadening effect

Apart from the frit-induced band broadening effect, various factors contribute to band broadening in CEC, including injection and detection (i.e. extra-column band broadening), eddy diffusion, molecular diffusion and mass transfer (i.e. intra-column band broadening) and Joule heating effect. In our case, since in-column and on-column detection have generated consistent peak efficiencies, the detection cell volume-induced band broadening is negligible. Meanwhile, based on the Waters Quanta 4000E CE system, a short UV slit of 1 mm was adopted and the time constant was always set to 0.1, therefore band broadening stemming from detection should be negligible.

In all the CEC experiments, electrokinetic injection was performed at 5 kV for 5 s. Using butylbenzene as the standard (retention factor ~1, as shown in Fig. 2), a butylbenzene band of around 2.5 s was loaded. Taking the CEC experiments at 20 kV for estimation (Fig. 2), the peak widths at half height for butylbenzene were measured to be 3.8, 3.4, 3.4, 3.6 and 3.9 s at detection points A, B, C, D and E, respectively. This reveals that the injection band width did contribute significantly to band broadening. Although Joule heating effect extensively takes place in CEC, it was not reported as a major contribution to band broadening. Rebscher and Pyell [30] compared packed capillary columns of 75, 100 and 150 μm i.d. for electrochromatography using high volume fraction of ACN (80–90%) in the separation electrolyte. The output currents were all below 10 μA and the Joule heating effect did not influence separation efficiency. In the present study, the currents measured were all below 4 μA. Under 20 kV, for instance, a current of 2 μA was recorded. Therefore, Joule heating-induced band broadening should be negligible in the present CEC conditions. As a result, apart from the major contribution from injection, the remaining band broadening should be mainly attributed to intra-column effect.

We further compared the present work with the abovementioned Rebscher and Pyell’s [6] (Section 3.2, Table 1), as both have used 3 μm C18 particle slurry-packed capillaries in in-column detection mode. Pyell et al. obtained the peak efficiency of ~120,000 plates/meter for bromobiphenyl, a retained aromatic compound. This efficiency is slightly lower than the efficiency (~150,000 plates/meter) that we observed for butylbenzene. Since 3 μm particles have been adopted in both studies (similar eddy diffusion term), the difference should be attributed to molecular diffusion and mass transfer of the analytes. Especially in the high speed range of van Deemter curve, the band broadening is mainly manipulated by mass transfer. Therefore, the major cause for this lower efficiency (~120,000 vs. ~150,000 plates/meter) should be attributed to mass transfer, which is dependent on retention factor. As evidenced in ref. [6], the retention factor for bromobiphenyl is over 1.5, while in our case, k for butylbenzene is around 1 (Fig. 2). The other factor is that bromobiphenyl has a lower diffusion coefficient than butylbenzene, this should also contribute to the mass transfer-induced band broadening.

3.4. Efficiency reproducibility

To confirm the consistency of peak efficiencies observed at different detection points around the column end area, another two transparent capillary columns of the same dimension as the above-investigated one (Section 3.2) were prepared. Using butylbenzene as the probe, peak efficiencies observed at the five detection points (points A, B, C, D and E, as in Fig. 1) were found to be consistent. For example, under 20 kV, relative standard deviations (RSDs) of plate heights recorded at the five detection points were 7.8% and 8.5%, respectively, on the two new columns. This result compares well with that of the first column, on which the RSD of plate heights of butylbenzene was found to be 7.9% between the five detection points. This series of reproducibility test confirms the observation of consistent peak efficiencies while the analyte band travelled through the single particle frit.

3.5. Considerations on optical path length, spatial resolution and general elution problem

In the in-column detection mode, the incident light goes through the packed bed rather than an empty tube. There is a large amount of stray light which do not go through the optical path length, this of course sacrifices the detection sensitivity. On the other hand, however, at the interfaces of the liquid and stationary phases, there is also diffuse scattering light which may in effect go through a longer path length than the capillary bore. Previous work [4,8,9] has proved that using silica-based packings running in high ACN content electrolytes, these two effects substantially compensated each other. This was also reflected in our experiments, where no significant peak height difference was sensed between in-column and on-column detection modes in a broad term (Fig. 2), although as specified earlier, there may be some evaporation-induced peak height fluctuations. The light scattering effect on the packing, however, leads to higher baseline noise. Banholzer and Pyell [9] and Chen and Horváth [31] respectively highlighted that the in-column detection normally generated baseline noise twice that with on-column detection.

It needs to be highlighted that, in-column detection should be better than on-column detection in terms of detection sensitivity, as has been proved in both photometric detection and fluorescent detection [8,9,32]. This enhancement effect comes from the “general elution problem”. Based on chromatographic retention, the
analyte band on the packed bed (in-column) is narrower than it is on an unpacked section (on-column) and this is manipulated by its retention factor ($k$) [32]. On the other hand, due to the significant difference in mobile phase fillable volumes between packed and open sections, there is also the “geometric focusing effect” in the open section, counteracting the “general elution problem” [8]. These factors have been combined in the equation Pyell et al. [8,9] derived to describe the signal enhancement effect between in-column ($S_i$) and on-column ($S_o$) detection: $S_i/S_o = (1 + k)^2/\varphi_M$, where $\varphi_M$ is the volume fraction of mobile phase in the packed column.

For a given column geometry, this enhancement effect is analyte dependent, i.e. the stronger the retention, the more significant the enhancement effect, as Banholzer and Pyell have corroborated [9]. In our case, since a high elution strength mobile phase (80% ACN) was used, in comparison to 70–60% ACN [9], a decreased retention (i.e. decreased $k$) was resulted, leading to insignificant enhancement effect. Meanwhile, in comparison with PAHs, the alkylbenzenes used in this study are not strongly retained species (e.g. $k$ for butylbenzene ∼1 in Fig. 2). Therefore it is not surprised that we did not sense the enhancement effect, while Yan et al. observed in their CEC experiments using PAHs [7]. It is well known that strongly retained species are poorly performed in terms of limit of detection in isocratic chromatography due to the general elution problem. Using in-column detection mode, the sensitivity enhancement effect should be advantageous in improving the limit of detection of such species.

Following the concerns over the general elution problem, Guthrie and Jorgenson [32] highlighted that the in-column detection should have a good spatial resolution. Nowadays, high spatial resolution is easily achievable by using narrow-width light beam. In our case, a UV beam width of only 1 mm has been used for in-column detection. Regarding the band widths of the analyte peaks (e.g. 5–6 mm for butylbenzene under 20 kV), it should provide enough spatial resolution.

3.6. Operational flexibility

As indicated by the efficiency data recorded at points C–E (Figs. 2 and 3), within 2 cm after the end frit (corresponding dead volume ∼160 nl), the high peak efficiency was well conserved. Previously, Adam et al. also reported a similar observation but on a thermo-immobilized packed capillary column [33]. The present observation suggests that even with certain on-column dead volume, the plug-like flow profile can be well conserved and molecular diffusion may not be significant over an on-column/pre-detection length of a couple of centimeters. This offers an important operational freedom for high efficiency microseparation followed by information-rich detection, as it allows placing multiple miniaturized detection units (e.g. UV, fluorescent detection etc.) in a tandem manner for comprehensive analysis of mass–limited complex mixtures.

4. Conclusions

In electrochromatography, it was deemed that in-column detection is better than on-column detection in terms of peak efficiency. In this study, robust and flexible capillary columns were fabricated using transparent capillary tube based on single particle fritting technology. Such columns enabled both in-column and on-column detection to be performed on the same capillary. The consistent van Deemter curves recorded at different positions around the column end indicated that, there was no significant band broadening while the analyte passed through the end frit. A comparison with previous studies has revealed that it was the single particle frit’s short length, unaffected surface and morphology that minimized the band broadening effect and consequently conserved the high efficiency. The demonstrated operational freedom of such columns should also be of usefulness in applications such as tandem detection for multi-parameter monitoring in analyses of complex mixtures, or on-line enzyme reaction assay demanding multipoint detection capacity [34]. The same single particle fritting technology has also been used to fabricate nanoCEC capillaries [18] and multi-column columns [35], further research into in-column and on-column detection for nanoCEC should be of significance in optimizing high resolution separations of mass-limited biomolecules.

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