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# Zero net-flow in capillary electrophoresis using acrylamide based hydrogel

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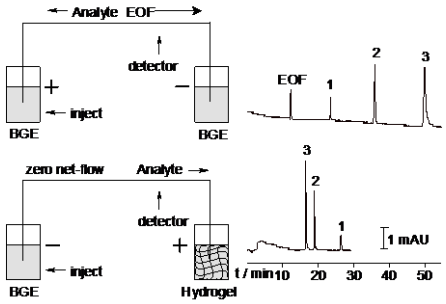


A simple approach to zero net-flow in electrophoresis using acrylamide based hydrogel at one end of the capillary is described.

**Abstract**

Zero net-flow was observed when acrylamide based hydrogel was used in a vial at one end of a fused-silica capillary during electrophoresis with electroosmotic flow. We demonstrate the detection of anionic compounds with the anode at the detector end and the field-enhanced sample injection of anionic small molecule drugs in counter-electroosmotic flow capillary zone electrophoresis.

The electroosmotic flow (EOF) is of fundamental importance, especially to electromigration techniques in capillaries[1–4] or microchips.[5,6] The EOF in fused-silica capillaries is provided by accumulation of cations at ionized silanol groups at the inner capillary wall. Under an applied electrical field, these cations migrate to the cathode and propel water from the bulk solution with a characteristic plug flow profile. In electromigration techniques, the EOF is typically controlled to improve on separation[7–16] and more recently on stacking or on-line sample concentration.[17–25] A common approach to control the EOF is by modifications of the chemical groups at the surface of the capillary wall by using dynamic or semi-permanent[7–13] and permanent[14–16] coatings.

Hydrogels are formed from the swelling of hydrophilic polymer networks due to the penetration of water into the network. The chemical and physical crosslinking prevents the polymers from dissolving while maintaining a high water content in the polymer structure. Hydrogels can be made to hold electrolytes by simply mixing the appropriate buffer with the monomers, crosslinker, and initiator before polymerization. In capillary electrophoresis (CE), a hydrogel coating has been used to manipulate the EOF.[26] A hydrogel which exhibits EOF was demonstrated for separation and biocatalytic applications that require passage of a solvent stream through the gel.[27] A hydrogel was used to encapsulate an enzyme for use as an integrated on-line enzyme reactor CE system.[28] Hydrogels were also simply added into the separation buffer as a dynamic modifier to reduce analyte adsorption effects and to enhance reproducibility and separation.[29-31] In microfluidic devices, hydrogels have been used mainly for preconcentration or enrichment[32-35] and also for separation.[36] In addition, a DNA-containing hydrogel plug immobilized in a microfluidic channel was demonstrated as a diagnostic microfluidic assay device via electrophoresis with a sacrificial fluorescent-tagged indicator oligomer.[37]

Closing one or both ends of the capillary is a possible approach to control the bulk flow inside the capillary. However, there are technical difficulties in closing the ends of the capillary, which could potentially prevent the electrical connectivity between the electrolytes in and outside of the capillary where the electrodes are located for electrophoresis. While this paper was being reviewed elsewhere, Oucacine and Taverna reported the use of 22% polyethylene oxide gel or viscous solution in a vial at one end of a capillary for the reduction of the apparent fluid flow in capillary isotachophoresis (cITP).[38]

Although hydrogels are mechanically strong, they also are soft and elastic. Thus, polyimide-coated capillaries and electrodes used in CE can easily penetrate hydrogels. In this work, hydrogels were made directly in CE vials by thermal polymerization of an aqueous mixture of acrylamide, *N*,*N*-dimethylacrylamide, potassium persulfate (initiator), and common electrolytes (i.e., sodium phosphate buffer). The hydrogel filled vial was used in lieu of the electrolyte filled vial in CE. The hydrogel physically blocked the anodic end of the capillary. This prevented the bulk liquid flow due to electroosmosis whilst maintaining electrical connectivity since the hydrogel can carry electrolytes in its water rich structure. The phenomenon of zero net-flow by acrylamide based hydrogel was demonstrated by the reversal of the electrophoretic migration order of anionic compounds in capillary zone electrophoresis (CZE) and the field-enhanced sample injection (FESI) of anionic small molecule drugs in counter-EOF CZE. In addition, the effect of hydrogel composition and CE electrolyte pH were studied.

Hydrogels were prepared directly in the 2 mL capacity CE vials by mixing 55%-wt acrylamide (monomer), 99% *N*,*N*-dimethylacrylamide (co-monomer), electrolyte stock solution, purified water, and 5%-wt potassium persulfate (initiator). The ratio of monomer, crosslinker

and initiator was 15:1:1.5. The other ratios are described in the text. The total volume was 1.2 mL. Aliquots of electrolyte stock solutions (e.g., 250 mM phosphate buffer, pH 7.4) and purified water were added to make up a final concentration of the total hydrogel volume similar to that of the separation or background electrolyte (BGE). The polymerization was thermally initiated at 60 $^{o}$C for 10 min. To remove excess reagents after polymerization, a small volume (e.g., 100 μL) of separation solution was placed into the vial and then was removed.

The CE experiments were performed on a Beckman P/ACE MDQ (Beckman-Coulter, USA) equipped with UV detector (214 nm) and 50 μm i.d. fused-silica capillary of 60 cm total and 50 cm effective length, respectively. The capillary was thermostated at 20$^{o}$C. New capillaries were conditioned by flushing (2 bar) with 0.1 N NaOH (30 min), followed by purified water (10 min), and separation electrolyte (10 min). Before each run, the capillary was flushed at 2 bar with 0.1 N NaOH (1 min), followed by purified water (1 min), and separation solution (5 min). All the steps described above were carried out without the placement of a hydrogel at the anodic end of the capillary. The experiments with hydrogel were performed without modification of the CE instrument. The CE procedure was basically the same without (see Fig. 1A) or with (see Fig. 1B) the use of hydrogel. The capillary was inserted directly into the vial containing hydrogel, as with regular CE. After each experiment with hydrogel, the capillary end was wiped with a damp tissue. The hydrogel vial can be used to as much as 5 CE runs. In the calculation of EOF, electrophoretic, and apparent electrophoretic velocities, the sign is negative and positive if electrophoresis was performed with anode and cathode at the detector end, respectively. The peak width was according to the method used by the United States Pharmacopeia (USP). The corrected peak widths were calculated by dividing the peak width by

the peak migration time. The LOD was estimated at a signal to noise ratio (S/N) of 3 based on peak height.

The counter-EOF CZE of pravastatin, indoprofen and tolfenamic acid using 100 mM phosphate at pH 7.4 and 20kV (normal polarity) as electrolyte and applied voltage, respectively is shown in Fig. 2A. Sample solution was 150 µg/mL of pravastatin (peak 1), 50 µg/mL indoprofen (peak 2), and 50 µg/mL tolfenamic acid (peak 3) prepared in the separation electrolyte. Injection was at 50 mbar for 5s with separation electrolyte at the capillary outlet end. The EOF velocity > electrophoretic velocity of the analytes, and thus the analytes were detected with the cathode at the detector end. The (apparent) EOF velocity was 5.45 cm/min while the electrophoretic velocity of pravastatin (peak 1), indoprofen (peak 2) and tolfenamic acid (peak 3) was -2.19, -3.00, -3.43 cm/min, respectively. The electrophoretic mobility of the EOF, peak 1, 2, and 3 was $1.64 \times 10^{-2}$, $-0.66 \times 10^{-2}$, $-0.90 \times 10^{-2}$, and $-1.03 \times 10^{-2}$ $cm^2/V*min$, respectively. The analytes were detected in the order of increasing electrophoretic mobility. The mobility was positive and negative if it was directed to the cathode and anode, respectively. The %RSD (n=3) for all the electrophoretic velocity and mobility values reported here were less determination were less than 0.5%.

Fig. 2B shows the same CZE experiment in Fig. 2A but with the hydrogel filled vial at the anodic and detector end of the capillary. The capillary was conditioned with the separation electrolyte but after sample injection, a reversed polarity at -20kV for separation was used. The electropherograms in the Fig. 2 were drawn in the same scale. The migration order in counter-EOF CZE reversed in B where the analytes were detected in the order of decreasing electrophoretic mobility (peak 3, 2, and then 1). In B, the apparent electrophoretic velocity of pravastatin, indoprofen, and tolfenamic acid was -2.05, -2.87, and -3.32 cm/min, respectively.

These values corresponded very well to the electrophoretic velocity obtained in the counter-EOF CZE (Fig. 2A). The electrophoretic mobility of peaks 1, 2 and 3 did not change ($-0.62 \times 10^{-2}$, $-0.86 \times 10^{-2}$, and $-1.00 \times 10^{-2}$ $cm^2/V*min$, respectively). The net-flow was then approximately zero in Fig. 1B or the EOF was cancelled as a result of the hydrogel. The magnitude of the hydrodynamic flow from blocking the end of the capillary is equal to the EOF, thus the net-flow was zero.[39] The gel-to-gel repeatability was briefly assessed. The %RSD (n = 4) for migration time, peak height and corrected peak area (corrected peak area = peak area/migration time) was 0.1 – 0.2%, 3.8 – 4.5%, 1.1 – 6.6%, respectively.

The effect of the hydrogel solidity on the net-flow was studied by the amount of monomer solution added in the hydrogel preparation. Eight hydrogels were prepared with 0.1 to 0.8 mL monomer solution (55 %-wt. acrylamide in water). The total volume of the hydrogel remained constant at 1.2 mL through compensation with the added amount of water. The hydrogel was a viscous liquid when 0.1 to 0.3 mL monomer solution was used. The hydrogel was solid when 0.4 to 0.8 mL was added. Using the same conditions as in Fig. 2B, zero net-flow was only achieved with solid hydrogels.

However, there was peak broadening with the use of a hydrogel in Fig. 2B due to the hydrodynamic flow generated by the blocking of the capillary. A circulating EOF that caused a pressure-induced or hydrodynamic back-flow in the middle of the channel.[39] Sharp peaks were, however, obtained by Oukacine and Taverna due to the focusing nature of cITP.[38] The extent of broadening or broadening factor was calculated by dividing the corrected peak widths obtained with the use of hydrogel by the corresponding widths without the hydrogel. A high value suggests stronger peak broadening due to the hydrogel. The broadening factor was 3.8, 2.7, and

2.4 for pravastatin, indoprofen and tolfenamic acid, respectively. The broadening caused a 50 to 75% decrease in peak height with the hydrogel.

It is well known that the EOF velocity increases with the increase in electrolyte pH. Small inorganic anions (bromide, nitrate, and bromate) with electrophoretic mobilities that are not significantly affected by changes in pH were analysed by counter-EOF CZE. The results at pH 3.0, 7.4, and 9.9 is shown in Fig. 3A, B, and C, respectively. Sample solution was 100 µg/mL of bromide (peak 1), 40 µg/mL nitrate (peak 2), and 400 µg/mL bromate (peak 3) in separation electrolyte. The sample was injected from the short end (10 cm to the detector) or cathodic end at 5 kV for 5s. Sample injection was performed without the use of a hydrogel filled vial. Voltage (-20 kV) was applied at reversed polarity because the electrophoretic velocities of the anions were faster than the EOF velocity. The EOF velocity was 2.08, 5.45, 6.54 cm/min at pH 3.0, 7.4, and 9.9, respectively. The EOF mobility was $0.62 \times 10^{-2}$, $1.64 \times 10^{-2}$, and $1.96 \times 10^{-2}$ $cm^2/V*min$, correspondingly. The injections were performed at the short end of the capillary (10 cm to the detector). The corresponding CZE with hydrogel in the anodic end is shown in Fig. 3D, 2E, and 2F. The migration times decreased due to the zero net-flow. More importantly in Fig. 3D, 3E, and 3F, the hydrogel consistently produced a zero net-flow at the studied pH range as indicated by the similar migration times for the analytes. The broadening factors were calculated as described above and the values were between 1.0 and 1.3. We also observed a 0 to 50% decrease in peak height with the hydrogel. These numbers are better than those obtained for the anionic drugs in the previous section. The decrease in peak height with the increase in pH of the separation electrolyte was probably due to the strength of the hydrodynamic flow from blocking the capillary with hydrogel. This flow was believed to be directly proportional to the

EOF velocity. A more detailed study on the hydrogel induced peak broadening by Taylor dispersion analysis will be performed in the future.

The repeatability of migration time, corrected peak area and peak height were calculated for the experiments using a hydrogel in Fig. 3 D – F. At pH 3.0 (Fig. 3D), the %RSDs (n=3) were 0.2 – 0.3%, 5.7 – 9.3%, and 3.2 – 8.4%, respectively. At pH 7.4 (Fig. 3E), the %RSDs (n=3) were 0%, 8.0 – 12.3%, and 7.3 – 10.3%. At pH 9.9 (Fig. 3F), the %RSDs (n=3) were 0 – 0.4%, 2.7 – 10.1%, and 1.8 – 3.0%, correspondingly. The USP resolution for peaks 1 and 2 and peaks 2 and 3 were calculated. The values at pH 3.0, 7.4 and 9.9 were 1.4 and 4.2, 1.8 and 3.9, and 1.9 and 4.1, respectively. These values were similar to those obtained from experiments without hydrogel. The USP plate numbers for peaks 1, 2 and 3 at pH 3.0 were 2448, 3457, and 4639, respectively. The numbers at pH 7.4 and pH 9.9 were 3210, 3681, and 4100, and 3569, 4212, and 4474, correspondingly. The plate numbers without hydrogel were not significantly different at 2424 to 8352.

According to Chien and Burgi, FESI of anions in counter-EOF CZE or FESI with polarity switching is performed as follows.[40] A water plug is injected hydrodynamically, followed by electrokinetic injection at negative polarity of the sample prepared in a low conductivity matrix. The sample is normally diluted with water. During injection, the EOF pushes the water plug into the inlet vial while the anions are introduced into the capillary with high velocities due to the enhanced electric field strength in the water zone. The water plug needs to be maintained inside the capillary during electrokinetic injection because the analytes are stacked between the water plug and separation solution zone boundary inside the capillary. This boundary moves with the same velocity as the bulk EOF. The measured current during injection is monitored closely, and when this current reaches around 98% of the current with

only separation electrolyte inside the capillary, the injection is stopped. This ensures that the stacking boundary is very close to the capillary inlet but is still inside the capillary. The stacked analytes are then analyzed by normal counter-EOF CZE with the separation electrolyte at each end of the capillary.

Here, a simple way to perform FESI without the need to monitor the current is demonstrated in Fig. 4B. For comparison, Fig. 4A is the counter-EOF CZE of anions similar to that in Fig. 2A except the sample diluent and separation electrolyte was 50 mM sodium phosphate at pH 7.4. It is noted that CZE separation in Fig. 4A and 4B were the same with the separation electrolyte at both ends and an applied voltage of 20 kV. Sample solution in Fig. 4A was 100 µg/mL pravastatin (peak 1), 50 µg/mL indoprofen (peak 2) and 50 µg/mL tolfenamic acid (peak 3) prepared in separation electrolyte. Sample solution in Fig. 4B was the sample in Fig. 4A which was diluted 500 times with purified water. The injection in (Fig. 4A was 50 mbar for 5s with separation electrolyte at the capillary outlet end and in Fig. 4B was -5 kV for 99s with hydrogel at the outlet. Also, a short water plug at 50 mbar for 3s was injected prior to sample injection in Fig. 4B.

In Fig. 4B, the hydrogel produced a zero net-flow and maintained the water plug and stacking boundary inside the capillary and close to the inlet end. The water plug was short and the measured current was around 98% of the current with only the separation solution. The high field strength was obtained at the tip of the capillary since only a small fraction of the capillary was filled with a low conductivity solution.[41] Comparison of the FESI injection with hydrogel (Fig. 4B) with the normal injection (Fig. 4A) shows that the use of the hydrogel afforded more than a 500-fold increase in peak signals. In Fig. 4B, the water plug which also served as EOF marker due to electrolyte discontinuity was also detected. The hydrogel which was at the other

end of the capillary was not responsible for the positive peak. In Fig. 4A, a negative peak that marked the EOF was observed due to the small amount of methanol which came from the sample stock solution. When FESI was performed without the hydrogel and the same conditions as in Fig. 4B, sample introduction was not achieved and no peaks were detected, as shown in Fig. 4C. It may be possible to obtain larger concentration factors but it is not attempted here.

It is emphasized that in order to maintain the water plug for at least 99 s at 5 kV injection, the net-flow must be zero which was achieved by the hydrogel. Another way to maintain the water plug is by applying a counter-pressure. We tried to apply pressure opposite to the EOF, however, the water plug was not well maintained. Another method is FESI with a longer water plug and current monitoring. The measured current during injection was monitored closely, and when this current reached around 98% of the current with only separation electrolyte inside the capillary, the injection was stopped. This ensured that the stacking boundary was very close to the capillary inlet but was still inside the capillary. The stacked analytes were then analyzed by counter-EOF CZE with the separation electrolyte at each end of the capillary. This procedure was difficult to automate and lower stacking efficiency was obtained because of the smaller enhancement in field strength during injection. A short water plug is required for greater enhancement in field strength as shown in Fig. 4B.

The use of acrylamide based hydrogel to produce zero net-flow in CZE with EOF was demonstrated. The approach is simple and can also potentially be used in electrophoretic microfluidic devices. A peak broadening effect that was possibly due to the hydrodynamic flow caused by the closure of the anodic end was directly proportional to the strength of the EOF. The strategy was successfully utilized for the fast separation of anions without significant loss in sensitivity and separation efficiency, as well as the efficient sample stacking using electrokinetic

injection under field-enhanced conditions of anions even under counter-EOF conditions. The zero net-flow in capillaries with hydrogel will be explored for other stacking systems, two-dimensional electroseparations, and electrokinetic sample microextraction/purification.

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**Figure captions**

Fig. 1. Schematic of normal counter-EOF CZE (A) and CZE with hydrogel at the anodic or outlet end of the capillary (B).

Fig. 2. Normal counter-EOF CZE (A) and CZE with hydrogel at the anodic or outlet end of the capillary (B). All electropherograms were drawn in the same scale and the inserted bar indicates the absorption value. The conditions are found in the text.

Fig. 3. Effect of EOF velocity by manipulation of pH on the CZE of small inorganic anions with hydrogel. Separation electrolyte was 100 mM sodium phosphate at pH 3.0 (A, D), 7.4 (B, E), and 9.9 (C, F). In (A-C), vials containing separation electrolyte were placed at both ends of the capillary. In (D-F), a hydrogel was placed at the anodic end of the capillary and separation electrolyte at the cathodic end. All electropherograms were drawn in the same scale and the inserted bar indicates the absorption value. The conditions are found in the text.

Fig. 4. FESI of anionic drugs in counter-EOF CZE without manual polarity switching. (A) is a conventional hydrodynamic injection. (B) is FESI with hydrogel. (C) is FESI without hydrogel. All electropherograms were drawn in the same scale and the inserted bar indicates the absorption value. Other conditions are found in the text.

Fig. 1

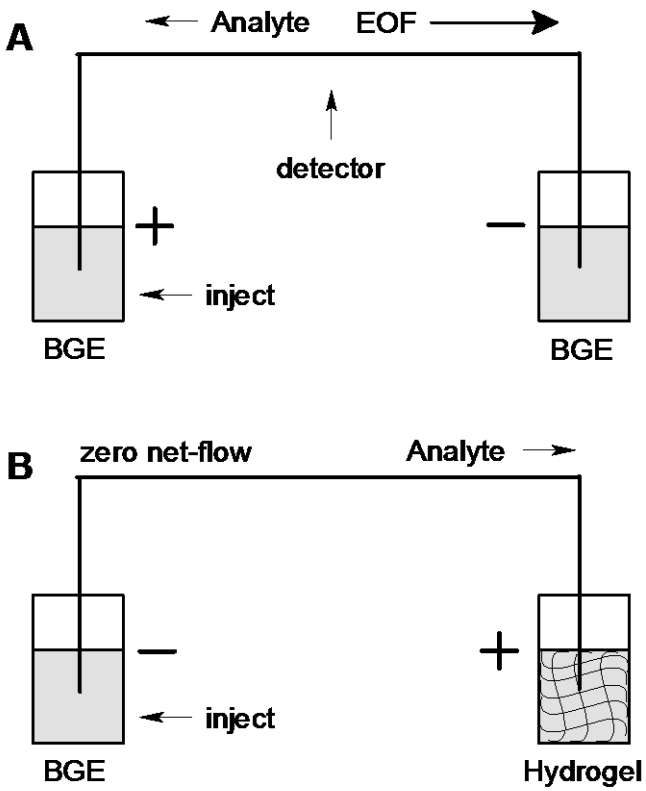

Fig. 2

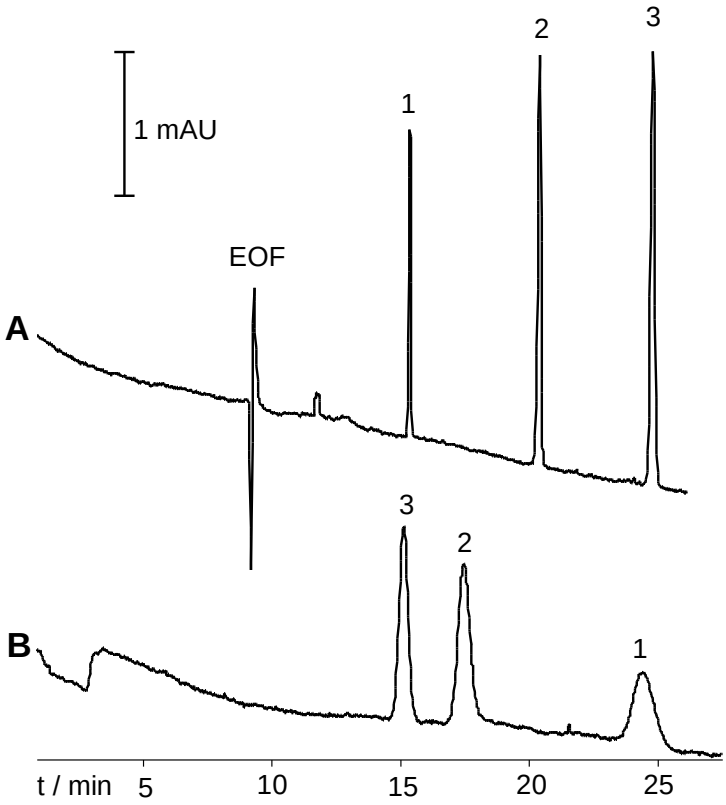

Fig. 3

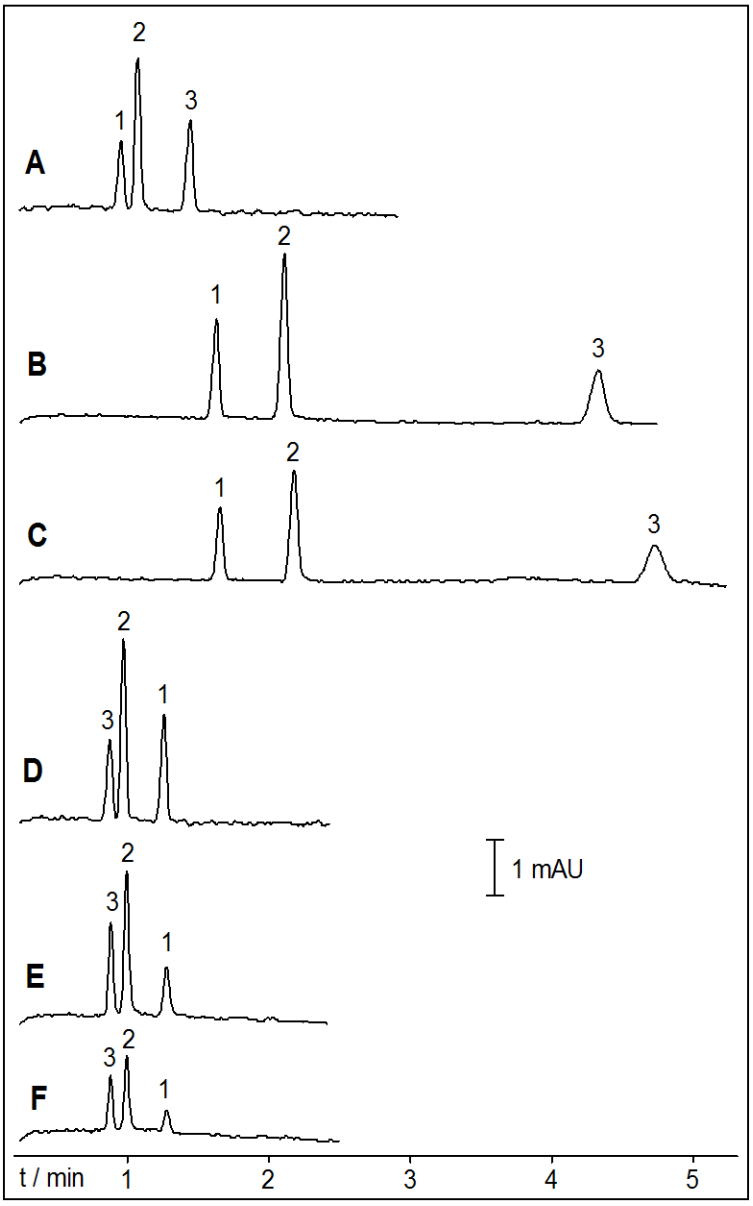

Fig. 4