

## New Strategy for On-Line Preconcentration in Chromatographic Separations

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### Abstract

In chromatographic separations the heights of peaks are proportional to the concentrations of sample components present in an injected mixture. In general, an increase in the peak height cannot be achieved by simply increasing the injection time or the sample plug length. An exception occurs if some form of on-line preconcentration is possible. We present a new strategy for achieving on-line preconcentration by the use of a porous chromatographic material that acts as a solid-phase extractor as well as a stationary-phase separator. We are able to realize significant on-line preconcentration using capillary columns filled with a photopolymerized sol-gel (PSG). More than 2-cm plugs of sample solution can be loaded into the capillary and concentrated using a running buffer that is the same as the injection buffer (to avoid solvent gradient effects). As a demonstration, mixtures of three different polycyclic aromatic hydrocarbons, eight different alkyl phenyl ketones, and five different peptides in solutions of aqueous acetonitrile have been injected onto the PSG column and separated by capillary electrochromatography. The preconcentration is marked in terms of peak heights, with up to hundred-fold increase for the PAH mixture, thirty-fold for the alkyl phenyl ketone mixture, and twenty-fold for the peptide mixture. Preconcentration takes place because of the high mass transfer rates possible in the highly porous structure, and the extent of preconcentration follows the retention factor  $k$  for a given analyte.

## **Introduction**

A common strategy for the analysis of trace species in complex mixtures is to preconcentrate the species of interest prior to separation. This procedure is most readily accomplished with some type of on-line preconcentrator. For example, in gas chromatography this goal is met by passing the gas stream through a cold column that is subsequently heated<sup>1</sup>. In high-performance liquid chromatography (HPLC) this process is usually done by gradient HPLC in which the analytes are retained on the column much more strongly for the first solvent than for succeeding ones<sup>2</sup>. On-line preconcentration has also enjoyed some success in electrophoretic separations. For example, in capillary zone electrophoresis Mikkers et al.<sup>3</sup> and Chien and Burgi<sup>4</sup> demonstrated that changes in electric field strength between sample and background solution zones can focus (stack) charged species, in electrokinetic chromatography Quirino and Terabe<sup>5,6</sup> have shown that micelles can act to concentrate (sweep) neutral and charged species, and in capillary electrochromatography (CEC)<sup>7</sup> Taylor et al.<sup>8</sup> reported that solvent gradients similar to gradient HPLC can concentrate neutral species. We describe here the ability of a photopolymerized sol-gel (PSG) capillary column (monolithic structure) to concentrate neutral and charged species in a liquid stream, which are subsequently separated by application of an electric field.

## **Methodology**

All electrophoresis experiments were performed with a Beckman P/ACE 2000 (Beckman Instruments, Fullerton, CA) equipped with fused silica capillaries (75- $\mu\text{m}$  inside diameter x 365- $\mu\text{m}$  outside diameter) purchased from Polymicro Technologies (Phoenix, AZ). The capillaries were thermostated at 20° C. Detection was done by absorption at 214 or 254 nm. Injections

were done using pressure (0.5 or 20 psi) or voltage (1 kV to 10 kV) and varied in duration from 2 s to 1920 s. UV irradiation at 365 nm of sol-gel solutions to form the PSG stationary phase was performed in a Spectrolinker XL-1500 (Spectronics Corp., Westbury, NY). Data analysis was performed with GRAMS/32 version 4.02 (Galactic Industries Corporation, Salem, NH). All electropherograms presented here were drawn to the same scale using GRAMS/32 software.

Unless stated, all reagents were purchased from Sigma-Aldrich (Milkwaukee, WI) in the highest grade available. The sol-gel solution was prepared as follows. A mixture of 375  $\mu$ l of tri-methoxy-silyl-(propyl)methacrylate (Gelest, Tullytown, PA or Sigma-Aldrich, Milkwaukee, WI) and 100  $\mu$ l of 0.12 M hydrochloric acid was stirred for 30 min at room temperature. 27 parts of this mixture were combined with 73 parts of toluene (porogenic agent) to give 200  $\mu$ l of the final solution. 5% by weight of the final solution of the photoinitiator Irgacure 1800 (Ciba Geigy, Tarrytown, NY) was added, and the resulting sol-gel solution was stirred for 5 min before use. The PSG column was prepared by filling a 75- $\mu$ m i.d. fused silica capillary with the sol-gel solution and exposing the column to UV light to affect photopolymerization. The polymerization length of the monolithic structure was controlled by removing a 15-cm stripe of the polyimide coating of the capillary prior to irradiation for 5 min. Unreacted reagents were flushed from the column with ethanol. The total length of the capillary was 25.6 cm (18.8 cm from inlet to the detector window). The detector window is positioned after the PSG material. The resulting PSG column is conditioned with the separation solution prior to use. The PSG structure acts as the chromatographic stationary phase<sup>9</sup>. Different selectivities can be achieved with the PSG phase by post modification with chlorosilane compounds containing various functional groups<sup>10</sup>.

For the peptide experiments the PSG column was prepared in the following way. A mixture of 575  $\mu\text{L}$  of tri-methoxy-silyl-(propyl)methacrylate and 100  $\mu\text{L}$  of 0.12 M hydrochloric acid was stirred for 30 min at room temperature. 20 parts of this mixture were combined with 80 parts of toluene to give 200  $\mu\text{l}$  of the final solution. The photoinitiator was added as 10% of the total volume of the final solution, and the resulting sol-gel solution was stirred for 5 min before use. The PSG column was prepared as described above. The column was rinsed with toluene. The PSG surface was modified by continuous flow of pentafluorophenyltrichlorosilane through the capillary for 45 min at room temperature and followed by rinsing with toluene.

For the alkyl phenyl ketone experiments the PSG column was prepared in the same manner as the column for the peptide experiments, except that the column was post-modified by continuous flow of (3,3,3-trifluoropropyl)trichlorosilane for 30 min at room temperature and followed by rinsing with toluene.

The samples were thiourea, naphthalene, phenanthrene, pyrene, acetophenone, propiophenone, butyrophenone, valerophenone, hexanophenone, heptanophenone, octanophenone, decanophenone, bradykinin, angiotensin II, tripeptide I (gly-gly-gly), tripeptide II (val-tyr-val), methionine enkephalin, hydrocortisone, progesterone, and cortisone. Stock solutions of thiourea, polycyclic aromatic hydrocarbons, alkyl phenyl ketones, and steroids were prepared in acetonitrile or aqueous acetonitrile. Stock solutions of test peptides were prepared in water. Stock solutions were mixed and then diluted prior to injection. Careful dilution of stock solutions with the separation solution components was undertaken to ensure that the sample matrix is identical to the separation solution to prevent gradient effects during the CEC experiments. The separation solution consisted of various portions of 50 mM ammonium acetate or 50 mM phosphoric acid, water, and acetonitrile. A new sample solution was used for every

injection to maintain the same concentration of acetonitrile in the sample solution and the separation solution. Other conditions can be found in the text or figure captions. As a precautionary note, proper care should be taken when handling the analytes, sol-gel reagents, and organic solvents as they are suspected to be health-threatening chemicals.

## Results and Discussion

*Effect of injection plug lengths on CEC peak shapes.* This work utilizes PSG columns without chromatographic particles. Figures 1a and 1b illustrate the increase in detection sensitivity with an increase in injected plug length in the CEC separation of the small molecule, thiourea, and three PAHs, and eight alkyl phenyl ketones. For the PAH mixture, the peaks are barely visible with the typical injection of a 0.1 mm plug length (Figure 1a, red); however, the peak heights increased when the plug length was increased to 6.8, 13.7, 27.4, and 34.2 mm (Figure 1a, blue, pink, green, and black, respectively). Similarly, for the alkyl phenyl ketone mixture, the peak heights increased when the plug length was increased from 0.7 mm (Figure 1b, violet) to 57.3 mm (Figure 1b, red). Note that for both mixtures the increase in peak height and the extent by which peak widths are narrowed are greater for the latter eluting compounds. The results suggest that the samples preconcentrate at the inlet of the CEC column, with the more retentive samples accumulating more than the less retentive ones.

In Figure 1a the improvement in peak heights for a 27.4-mm injection (green) compared to a typical injection of 0.1 mm (red) is 50, 125, and 127 times for naphthalene, phenanthrene, and pyrene, respectively. Note that the sample solution in the green electropherogram is a 10-fold dilution of the sample in the red electropherogram. In Figure 1b, the improvement in peak heights for a 28.6-mm injection (acetophenone, propiophenone, butyrophenone, and

valerophenone) (blue) and 57.3-mm injection (hexanophenone, heptanophenone, octanophenone, and decanophenone) (red) compared to a typical injection of 0.2 mm (not shown) is 13, 15, 19, 21, 20, 26, 28, and 32 times greater for the alkyl phenyl ketones (in the order of increasing alkyl chain length).

***Evidence of preconcentration in CEC with PSG.*** Figures 2 and 3 provide proof that preconcentration takes place in the PSG columns. The height of a chromatographic peak is proportional to the concentration of a sample component. Figure 2 illustrates that peak heights are constant for a given concentration of sample unless there is a preconcentration process. In general, with increasing injection plug lengths, peak heights remain constant while peak areas and peak widths increase linearly. Figure 3a shows an electrochromatogram of a small plug injection of naphthalene prepared in the separation solution. Figure 3b is an electrochromatogram obtained from a ten-fold dilution (in the separation solution) of the naphthalene solution used in Figure 3a but injected as a longer plug. The corrected peak areas (peak area / migration time) for both electrochromatograms are made close to each other by controlling the injection time of the ten-fold dilution of sample. The corrected peak areas of the electrochromatogram in Figure 3a and 3b are 0.0023 (%RSD=0.02%, n=3) and 0.0025 (%RSD=0.00, n=3) arbitrary units/min, respectively. This comparison was done such that the amount of naphthalene molecules injected for each run is the same.

Preconcentration is evident because the peak height is slightly higher for the longer injection of diluted sample while the corrected peak widths (peak width / migration time) for both experiments are almost the same, although the sample concentrations are different. The peak heights of the electrochromatograms in Figure 3a and 3b are 0.0869 (%RSD=0.36%, n=3)

and 0.0937 (%RSD=0.06%, n=3) arbitrary units, respectively. The peak widths of the electrochromatograms in Figure 3a and 3b are 0.0253 (%RSD=0.07%, n=3) and 0.0249 (%RSD=0.01%, n=3) arbitrary units/min, respectively. The shift in migration time on Figure 3b is caused by the longer injection time, which makes the center of the sample plug closer to the detector window.

***Application to quantitative analysis.*** The limit of detection (signal/noise = 3) for a typical 0.1 mm injection plug is 1.5 mM, 0.2 mM, and 1.2 mM for naphthalene, phenanthrene, and pyrene, respectively. The limit of detection (signal/noise =3) for a 27.4-mm injection plug using 0.5 psi is 24.3  $\mu$ M, 5.4  $\mu$ M, 3.4  $\mu$ M for naphthalene, phenanthrene, and pyrene, respectively. The correlation coefficient ( $r^2$ ) is > 0.99; the reproducibility of migration time is less than 2% RSD (n=7); and the reproducibility of peak height is less than 8% RSD (n =7). This data suggest a one to two orders of magnitude increase in concentration detection sensitivity compared to a typical injection.

The PSG column with on-line preconcentration may also be useful for semi-preparative purposes, which is now being explored in our laboratory. More than 100 nl of sample solution at analyte concentrations in the mM levels can be injected into the column without significant deterioration of peak shapes. Moreover, the use of solvent gradients improves detection sensitivity by a factor of two above and beyond the results presented here.

***Application to peptides.*** Figure 4 shows the separation and preconcentration of five peptides in a PSG column modified to contain a pentafluorophenyl moiety. Note here that the cathode directed velocities of the peptides are dictated by both electrophoretic and electroosmotic flow effects. The peptides have a net positive charge at the pH of the separation

solution (pH ~2). The improvement in peak heights for the longer injection (Figure 4b, red) compared to a typical injection (Figure 4a, blue) is 21, 19, 16, 18, and 22 times for bradykinin, angiotensin II, tripeptide I, tripeptide II, and methionine enkephalin, respectively. The sample solution in both electropherograms is the same. Note that the enrichment factors for the first two eluting compounds (bradykinin and angiotensin II) are slightly higher than the next two compounds (tripeptide I and tripeptide II). The affinity of bradykinin and angiotensin II to the modified PSG phase must be greater than that of the tripeptides.

***Application to real analysis.*** A urine sample, spiked with hydrocortisone, progesterone, and cortisone, is evaluated with the PSG column (Figure 5). These steroids after protein precipitation of urine sample with acetonitrile are easily detected with a 21.4-mm injection of the resulting sample solution (Figure 5b), but are weakly detected with a typical injection (Figure 5a). To prevent solvent gradient effects, the resulting sample solution was made to contain the same amount of acetonitrile as the separation solution. Electrophoretic preconcentration effects caused by the presence of salts in the resulting sample solution is also not possible because the steroids are neutral and thus unaffected by an electric field. A comparison between the blank run (Figure 5c) and the spiked run (Figure 5b) show that the sample matrix, which still contains other biomolecules, did not significantly interfere with steroid separation on the PSG column. This demonstrates the usefulness of the strategy for biofluid analysis.

***Preconcentration mechanism.*** Highly porous structures like the PSG material used in this study have high rates of mass transfer and low resistances to flow.<sup>11</sup> The high mass transfer rates arise from the enhanced accessibility of the analytes to the binding sites of the porous structure. This behavior is similar to high mass transfer rates observed in monolithic stationary phases<sup>11</sup> and in perfusion chromatography.<sup>12</sup> Because of the high mass transfer rates, the kinetics



of analyte-PSG interaction (i.e., the partitioning of the analyte between the mobile and stationary phases) is not the rate-limiting step in the separation. Consequently, it is possible to inject and concentrate larger volumes of sample solution than in columns containing normal chromatographic materials.

It is useful to introduce the retention factor  $k$ , which is the ratio of the number of moles of solute in the stationary PSG matrix to that in the mobile phase, to describe the separation process. We carried out measurements that showed that the value of  $k$  is unchanged for electrically neutral solutes separated by pressure or by voltage.<sup>9</sup> Moreover, we found that the flow rate hardly influenced the extent of preconcentration. Consequently, we conclude that the total preconcentration effect is directly proportional to the  $k$  value, with the longer injection plug lengths (e.g., > 25 mm) leading to severe peak broadening of analytes having low- $k$  values (thiourea and naphthalene in Figure 1a, black; and thiourea, acetophenone, propiophenone, butyrophenone, valerophenone, and hexanophenone in Figure 1b, red). This behavior implies a maximum length of sample plug for each analyte before peak shape becomes compromised. Thus, the porous structure created in photopolymerized sol-gel acts to extract the analytes from solution as well as provides the stationary phase for chromatographic separation of the analytes. It is this extractor-separator combination that gives this method such power.

The preconcentration effect should occur to some extent in other CEC studies, but to our knowledge has not been previously reported. Recent studies by Zhang et al.<sup>13</sup>, Hilhorst et al.<sup>14</sup>, and Chen et al.<sup>15</sup> under nongradient conditions in packed CEC columns actually showed no substantial increase in peak heights with longer than typical sample injections.

## Conclusion

A new strategy for sample preconcentration is described in which the column acts simultaneously as a solid-phase extractor as well as the stationary phase for chromatographic separation. Preconcentration occurs in a porous structure that is capable of rapid mass transfer allowing for the injection of large volumes of sample solution. Preconcentration is dependent on the retention factor  $k$  for a given analyte. Applications to various samples have been demonstrated using monolithic columns filled with photopolymerized sol-gel (PSG), and the preconcentration effects achieved have been sizeable. This method of preconcentration is a general one and other porous structures in addition to PSG are expected to show this feature.

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**Figure 1.** Electrochromatograms of the separation of (a) thiourea (peak 1; 12.5  $\mu\text{M}$ ), naphthalene (peak 2; 51.0  $\mu\text{M}$ ), phenanthrene (peak 3; 1.0  $\mu\text{M}$ ) and pyrene (peak 4: 123  $\mu\text{M}$ ); plug lengths, red = 0.1 mm, blue = 6.8 mm, pink = 13.7 mm, green = 27.4 mm, black = 34.2 mm; sample and separation solution, 50 mM ammonium acetate/water/acetonitrile (1/4/5); applied voltage, 20 kV; detection, 214 nm; (b) thiourea (peak 1; 5  $\mu\text{M}$ ), acetophenone (peak 2), propiophenone (peak 3), butyrophenone (peak 4), valerophenone (peak 5), hexanophenone (peak 6), heptanophenone (peak 7), octanophenone (peak 8), and decanophenone (peak 9); plug lengths, violet = 0.7 mm, orange = 7.2 mm, green = 10.7 mm, black = 17.9 mm, blue, 28.6 mm, red, 57.3 mm; sample and separation solutions are the same as in (a); applied voltage, 15 kV; detection 254 nm; the concentration of each of the alkyl phenyl ketones is 0.1  $\mu\text{g}/\text{mL}$  in the separation solution.

**Figure 2.** Expected peak shapes for sample prepared in the separation solution injected as (a) short or (b) long plug.

**Figure 3.** Electrochromatograms showing the 1 kV injection at (a) 5s of a 39.0 mM and (b) 85 s of a 3.9 mM of naphthalene in the separation solution; sample and separation solution, 50 mM ammonium acetate/water/acetonitrile (1/3/6); applied voltage, 15 kV; detection, 214 nm.

**Figure 4.** Electrochromatograms showing the 0.5 psi injection at (a) 0.1 and (b) 12 mm of test peptides in a matrix having the same composition as that of the separation solution; test peptides, bradykinin (peak 1), angiotensin II (peak 2), tripeptide I (peak 3), tripeptide II (peak 4), and methionine enkephalin (peak 5); peptide concentrations, 16.7  $\mu\text{g}/\text{ml}$  each; separation solution, 50 mM phosphoric acid/water/acetonitrile (1/5/4); applied voltage, 15 kV; detection, 214 nm, 20°C.

**Figure 5.** Electrochromatograms showing the injection at (a) 0.1 mm and (b) 21.4 mm of urine spiked with hydrocortisone (1), progesterone (2), cortisone (3), and (c) 21.4 mm of urine blank; sample preparation, spiked urine contained 0.1 mM of 1, 0.3 mM of 2, and 0.2 mM of 3, 4 parts of spiked or unspiked urine was mixed with 6 parts of acetonitrile and centrifuged to remove the proteins, 1 part of each supernatant was mixed with one part of 50 mM ammonium acetate/water/acetonitrile (1/7/2) before injection; separation solution, 50 mM ammonium acetate/water/acetonitrile (1/5/4), applied voltage, 17 kV; detection, 254 nm.

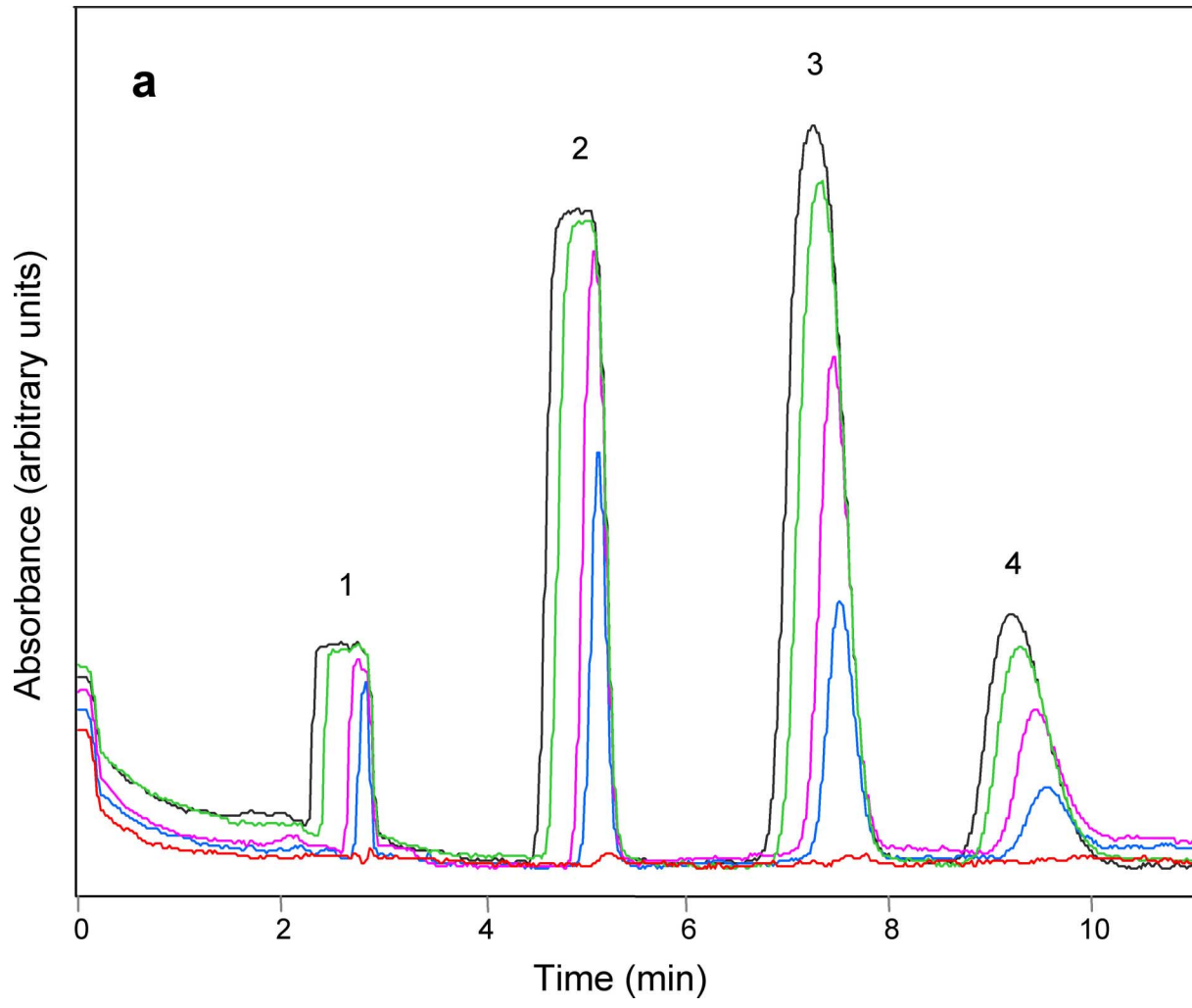


Fig. 1a

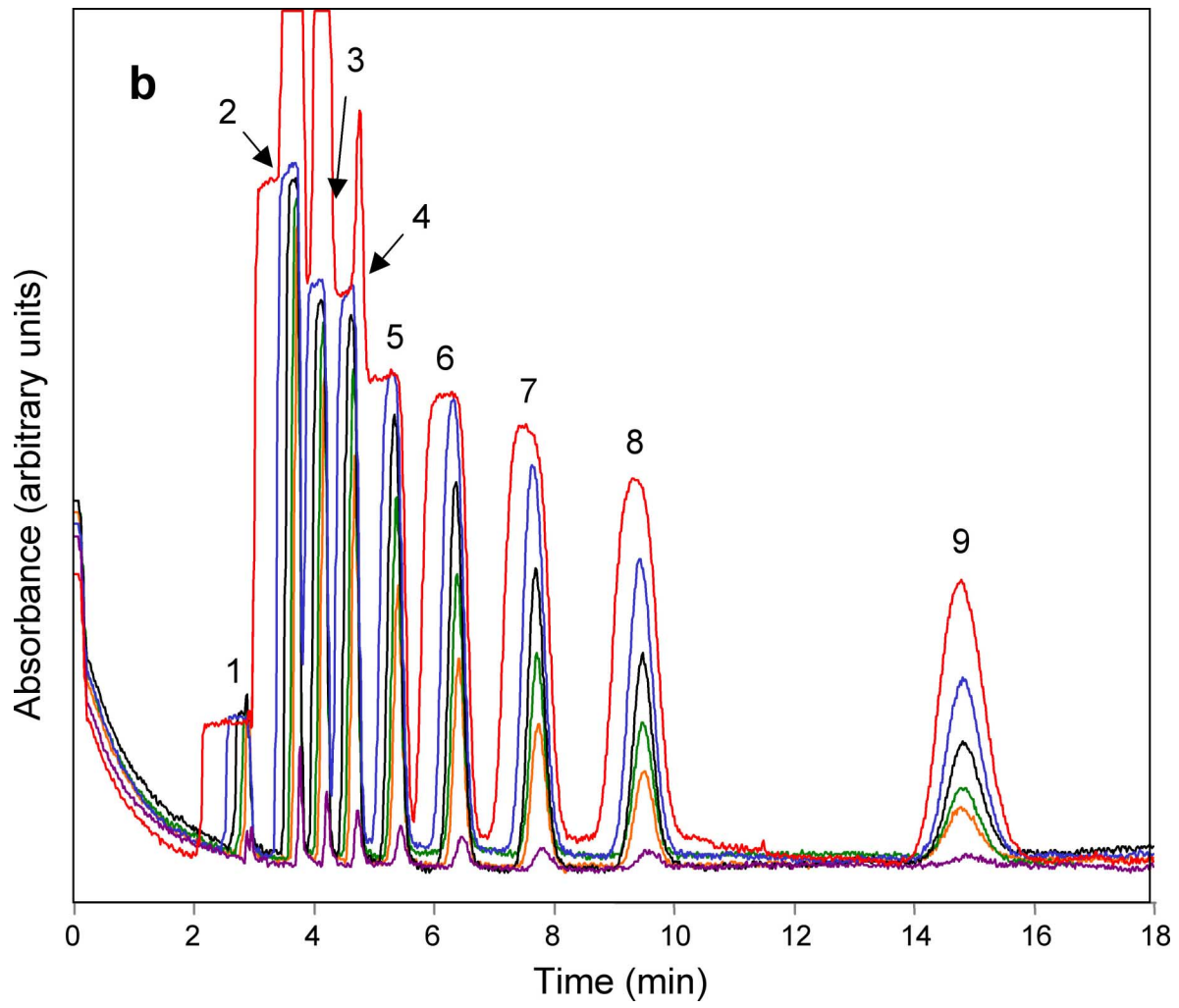


Fig. 1b

**a**, typical short injection



**b**, long injection



Fig. 2



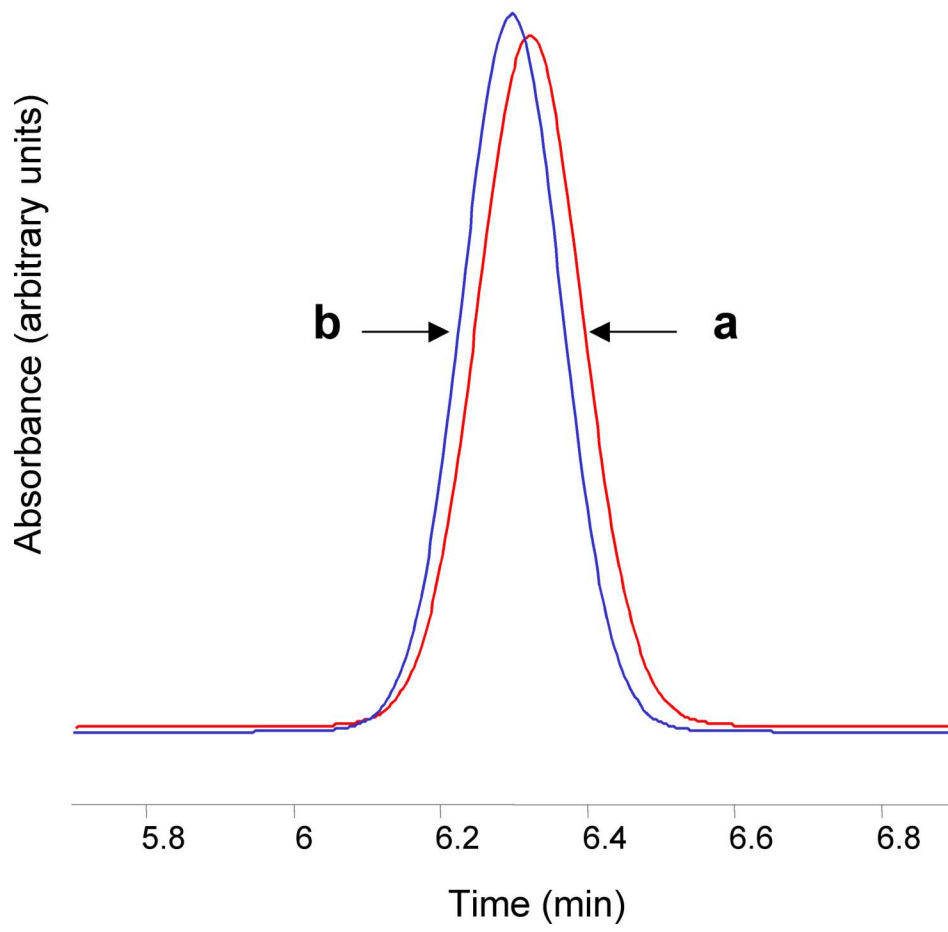


Fig. 3

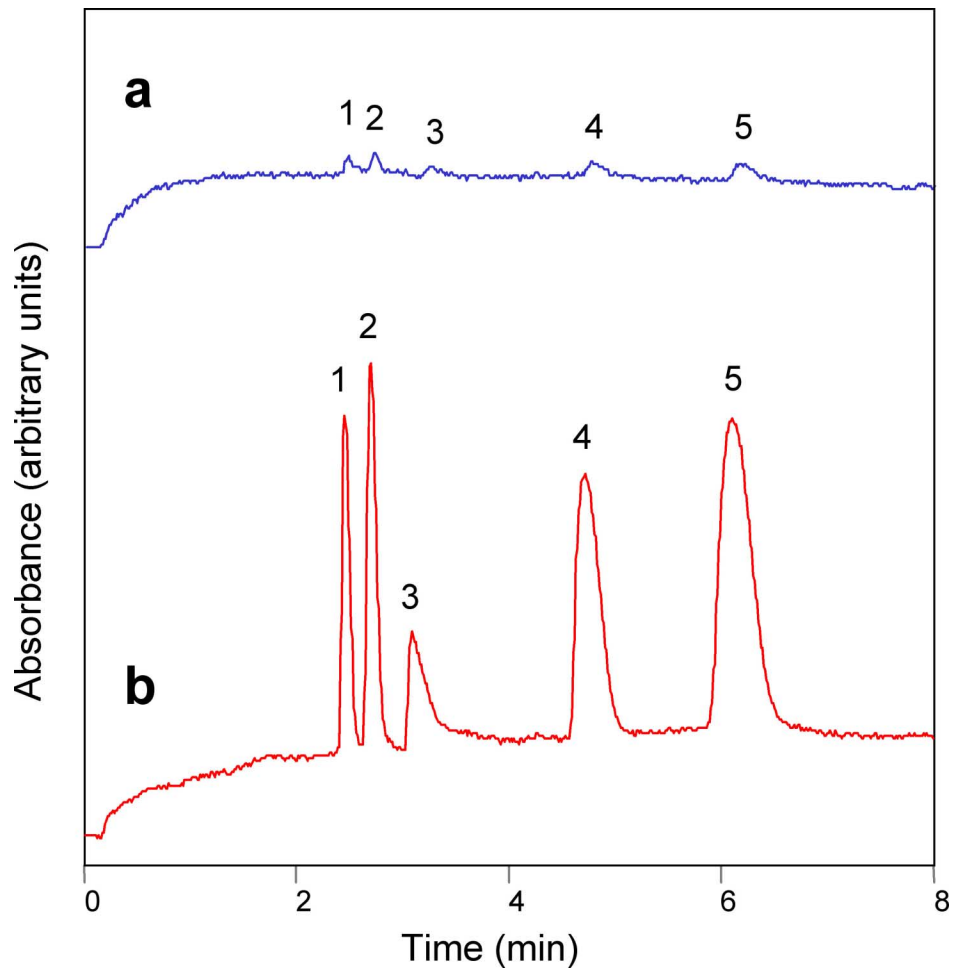


Fig. 4

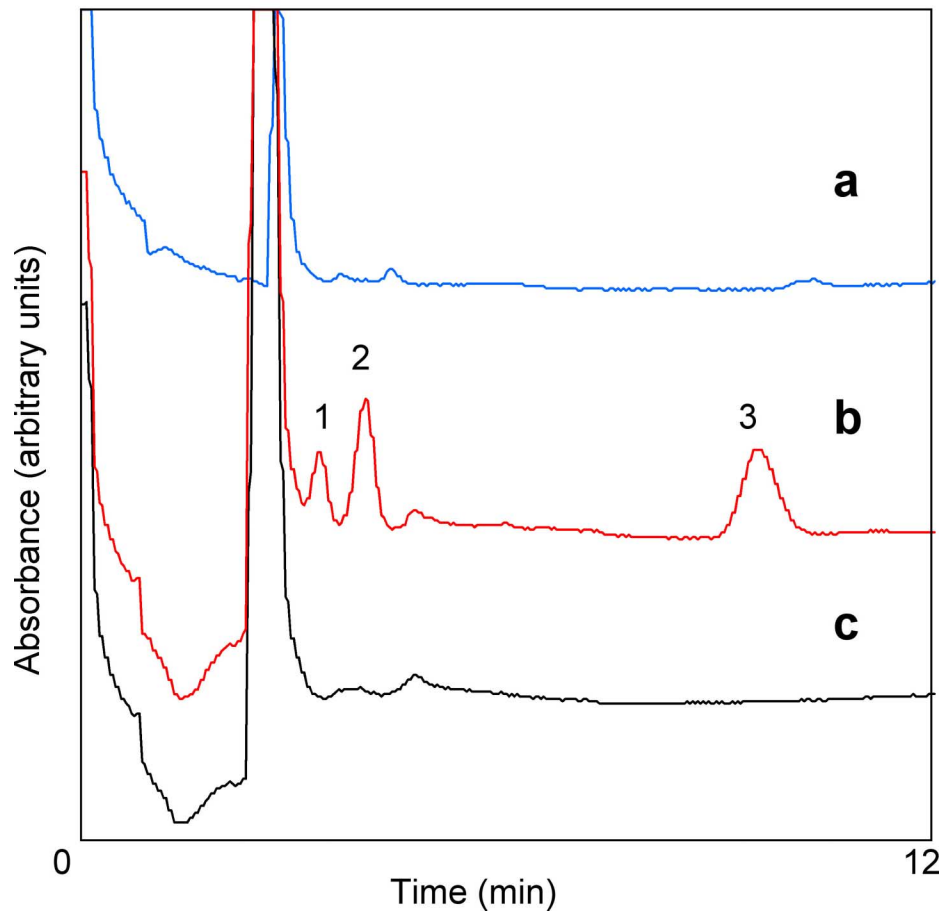


Fig. 5