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Two variables dominating the retention of intact proteins under gradient elution with simultaneous ultrafast high-resolution separation by hydrophobic interaction chromatography

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Abbreviations

1D-LC	one-dimensional liquid chromatography
2D column	two-dimensional column
2D-LC	two-dimensional liquid chromatography
2D-LC-1C	2D-LC carried out with only a single column
$C_{(i,D)}$	instantaneous displacer concentration
C_{CMP}	displacer concentration at the critical migration point
CIM®	monolithic disks with extremely large pores suitable for the fast separation of proteins
CMP	critical migration point
C_{CMP}	concentration corresponding to the critical migration point
C'_{CMP}	operation C_{CMP}, $C'_{CMP} = C_{CMP} + W(M)$
CC	conventional chromatography
HIC	hydrophobic interaction chromatography
HPLC	high performance liquid chromatography
i.d.	internal diameter
IEC	ion exchange chromatography
k	isocratic elution retention factor
k^*	gradient elution retention factor
L	column length or thickness of the chromatographic cake
L_{min}	minimum column length or minimum thickness of the chromatographic cake
LC	liquid chromatography

mD-LC	multidimensional liquid chromatography
MR	migration region
MS	mass spectrometry
n_i	number of samples during the i^{th} nonsynchronous sampling step
PC	partition coefficient
PPC	precipitation chromatography
R	linear correlation coefficient
$R_{r(S/M)}$	ratio of the chromatographic resolutions in the steady and migration regions, $Rr_{(S/M)} = Rs_{(R,S)}/Rs_{(R,M)} = \Delta T_{(R,S)}/\Delta T_{(R,M)}$
R_s	chromatographic resolution for protein separation
$R_{s(R,S)}$	chromatographic resolution of two adjacent peaks arising from their respective steady regions
$R_{s(R,M)}$	chromatographic resolution of two adjacent peaks arising from their respective migration regions
$R_{t(S/M)}$	ratio of retention times in the steady and migration regions, $(T_{(R,S)}/T_{(R,M)})$
$R_{t(M/R)}$	ratio of the retention time in the migration region over the real retention time $(T_{(R,M)}/T_R)$
RPLC	reversed-phase liquid chromatography
SR	steady region
SSP	solubility of the synthetic polymer
t_{inj}	injection time

T_0	column dead time
T_{CMP}	time corresponding to the critical migration point, $T_{CMP} = T_0 + T_{(R, S)}$
T_i	actual time of gradient elution
T_R	real retention time of the protein, $T_R = T_{(R,M)} + T_{(R,S)}$
t_R	total retention time of the protein, $t_R = T_0 + T_{(R, S)} + T_{(R,M)}$
$T_{(R,avg)}$	average retention time
$T_{(R, i)}$	retention time corresponding to the i^{th} sampling
$T_{(R, i+1)}$	retention time corresponding to the $(i+1)^{th}$ sampling
$T_{(R, i-1)}$	retention time corresponding to the $(i-1)^{th}$ sampling
$T_{(R,M)}$	retention time in the migration region
$T_{(R,S)}$	retention time in the steady region
WCX	weak cation-exchange chromatography
W_A	width of peak A
ΔS_R	distance between two adjacent peaks
$\Delta T_{(R,S)}$	difference in the retention times at which two adjacent steady-region peaks occur
$\Delta T_{(R,M)}$	difference in the retention times at which two adjacent migration-region peaks occur
σ	standard deviation

Abstract: The retention of intact proteins under gradient elution in hydrophobic interaction chromatography (HIC) was found to be governed by two variables of the steady region (SR) and migration region (MR). In the SR, the proteins are immobilized by the strong interactions with the stationary phase such that the retention time is independent of the column length. In the MR, the proteins also interact with the stationary phase, but they move normally, thus, the retention time depends on their partition coefficients and the column length. The SR can be used as an operation space (OP) for high-throughput protein analysis by 1D-LC using short columns under high flow rates to maintain a high resolution. The OP can also be employed for all assisted operations in online 2D-LC. Based on the steady region/migration region optimization strategy developed in this study, five successive complete separations of seven intact proteins were performed in a HIC cake in less than 5 min, and a crude extract of ribonuclease A from bovine pancreas was purified using online 2D-LC to 95.8% purity with 93.2% mass recovery in 45 min. This approach can be used to expedite the purification of drug-target proteins and should therefore be of interest to the pharmaceutical industry.

Keywords: intact protein, gradient elution, proteomics, continuous ultrafast analysis, high resolution, online operation space

1. INTRODUCTION

Based on traditional column chromatography that was invented by Tswett in 1903, [1] nonporous particles, [2-5] monolithic columns, [6-9] high temperatures, [10-16] and sub-3 μm core-shell particles [17-19] have been used to accelerate the separation of solutes in modern high-performance liquid chromatography (HPLC) since the 1960s.[20] However, the separation speed depends on the complexity of the sample. Whether one-dimensional (1-D), two-dimensional (2-D), or multidimensional (mD) liquid chromatography (LC) is used depends upon the need for fast separation, high resolution, or a compromise between the two. [2, 21-29]

This choice is particularly important in developing proteomics in which intact proteins are fragmented directly in the mass spectrometer to achieve both protein identification and characterization, even capturing information on combinatorial post-translational modifications. [30] It was recently reported that the main problem with "bottom-up MS" strategy of that " it is no longer the actual biological actors but the pieces left after they've been broken.[31] "Top-down MS" strategy has many advantages and holds tremendous potential for characterization and quantification of intact proteins and it has emerged as an alternative to common "bottom-up strategies" for protein analysis. Especially, the improvements in MS, which have allowed measurement of molecular masses of up to 229 kDa have expanded the application range of the "top-down MS" strategy.[32] Neil L Kelleher et al improved on previous top-down studies and employed four types of separations in series when they identified more than 1,000 human gene products that were posttranslationally modified in different ways, resulting in a total of more than 3,000 protein species.[33-35]

With an interface transfer between two columns, both off-line and on-line manners can be adopted, the former can operate large sample, but very slowly; while the latter is very fast but only can transfer volume solution in μL scale with accounts of 1/10 to 1/100 of the fraction from the previous separation mode. Thus, the "top-down" MS strategy requires further solve these problem of high sample size, high resolution, and high speed 1D-LC and mD-LC), requiring operating milligram-scale sampling of intact proteins. [36,37] Top-down MS and proteomics would benefit from further improvements in speed, purity and mass recovery or from the improvement of all three together.

The use of high temperature, [10-16] nonporous particles, [2-5] monolithic separation media, [6-9] and sub-3μm core shell particles[17-19] have been reported to accelerate separation. However, Wang *et al*. recently reported that column regeneration and re-equilibration usually requires much more time than the chromatographic separation step itself. [2] For example, Roth et al. employed superficially porous resin and an online capillary for intact LC/MS protein analysis in whole-cell extracts.[38] The fast separation in LC inevitably compromises the resolution of the measurements. Stoll et al. have summarized cutting edge developments in the field and predict the separation speeds that can be achieved for peptides without sacrificing resolution.[26]

Gradient elution has been employed to quickly separate substances whose migration can be modified by shortening the gradient elution time and/or increasing the steepness of the gradient. [39-42] Gradient elution includes both conventional liquid chromatography in which the separation mechanism for substances is dominated by the partition coefficient (PC) of the substance and is described using plate theory, [41,43] and it also includes

non-conventional liquid chromatography, or precipitation chromatography (PPC), in which the solubility of synthetic polymers (SSPs) dominates the retention and does not follow plate theory. [42] As a result, the effect of the column length on the chromatographic resolution for a substance is quite different, and the results conflict each other in the literature. [44-49]

Although many different retention mechanisms for substances were reported in HIC, [50-55] reversed phase liquid chromatography, [56-61] and unified retention models, [62,63] they all employed the retention factor *k* in isocractic elution to express the retention character and the gradient retention factor *k** in gradient elution, which is defined as the medium value of *k*. Both *k* and *k** are single variables obtained from experimental results.

The main advantage of hydrophobic interaction chromatography (HIC) is that the chromatographic conditions are very close to the physiological conditions of the human body, such as a neutral pH, an aqueous salt solution, and room temperature, all of which are favourable to the maintenance of the proteins bioactivity. [64] HIC has been also used to demonstrate the utility of HIC. This is the case in drug biotechnology for assessing the heterogeneity stability and, in some cases, potency of monoclonal antibodies. The separate antibodies differ by as little as 4 kDa, [65] and for the refolding of denatured proteins. [23,66-67]

The conventional purification method for protein drugs that originate from biotechnology or animal organs may feature a precipitation step for coarse separation and subsequently several fine LC purifications that take a long time and gives a low recovery. [68-71] When a chromatographic cake was employed that is larger in diameter than in length/thickness, the separation speed was reported to increase.[23,72] RNase A is a

well-characterized enzyme that hydrolytically cleaves the phosphodiester backbone of RNA [73,74] and can be used as an anticancer chemotherapeutic agent. [75] The enzyme can be isolated from bovine pancreas,[69] but the most efficient method for its purification is offline 2D-LC, which has the same limitations as those discussed for the isolation of rsCD14. [71]

The purpose of this study is to establish a new method for exploring the ultrafast high-resolution separation of intact proteins by LC. The method bases on the found the retention character of intact proteins in the stationary phase during HIC separation under gradient elution to compose of “steady region” (SR) and “migration region” (MR). In the SR, the protein retention does not follow plate theory and retention time is independent of column length. In the MR, the retention follow plate theory and retention time depends on their partition coefficients and column length.

We have developed a method for the ultrafast high-resolution separation of intact proteins. This technique utilizes the combined exploitation of the SR and MR, allowing separation under a very high flow rate on a suitably sized chromatographic cake in which very small particles are packed. Using this HIC cake, a satisfactory separation of seven intact proteins was obtained in 5 min by 1D-LC, and a crude ribonuclease A extract from bovine pancreas was purified in 45 min by online 2D-LC to 95.8% purity with a mass recovery of 93.2% using a single column.

The SR may also be utilized as an operation space for online operations. This would allow for more rapid analysis, but can also expedite purification and production of protein-based drugs in a pharmaceutical context.

2. EXPERIMENTAL

2.1. Apparatus

A LC-10A high-performance liquid chromatograph (Shimadzu, Kyoto, Japan) consisting of two pumps (LC-10ATvp), a variable wavelength UV-Visible detector (SPD-10Avp), a system controller (SCL-10Avp), a column oven (CTO-10ASvp), a degasser (DGU-12A), and a chromatographic workstation (Class-vp) were employed. The system is an improvement over the system reported in a previous paper.[76] A HIC column (TSKgel Ether-5PW, *L* × i.d.: 75 mm × 7.5 mm, Tososh Co., Tokyo, Japan) and a HIC chromatographic cake (*L* × i.d.: 4 mm × 10 mm, Xi'an Aolan Science and Technology Development Co., Xi'an, China, http://www. Aulast.com) were packed with TSKgel Butyl-NPR (Tososh Co., Tokyo, Japan) as specified in the literature. [27] A silica-based 2D (WCX, HIC) column (50 mm, x 4.6 mm i.d., 5 μm, 22 nm) was obtained as a gift from Xi'an Aolan Science and Development Technology Co. (Xi'an, China, http://www. Aulast.com).

HPLC-grade water was prepared in a Cascada LS system（Pall Co. Ltd, USA. A UV spectrometer (type 1601PC) was from Shimadzu Co (Kyoto, Japan) and an electrophoresis system DYY-6C made in Beijing 61 Co. (Beijing, China). The employed centrifuge (Sorvall Evolution RC, 20,000 rpm) was from Sorvall Co. (Asheville, NC, USA), a dual wavelength flying spot scanning aensitometer (CS-9301PC) from Shimadzu Co.(Kyoto, Japan) and a pH meter (PB-10) from Sartorius, CA, USA).

2.2. Chemicals

Cytochrome C (Cyt C, horse heart, type III), myoglobin (Myo, horse heart), ribonuclease A (RNase A, bovine pancreas, type I-A), α-chymotrypsin (α-Chy, bovine pancreas), lysozyme (Lys, chicken egg white), α-amylase (α-Amy, Bacillus anthracis type IIA), bovine serum albumin (BSA, bovine pancreas), and insulin (Ins, bovine pancreas) were purchased from Sigma Co. (St. Louis, MO, USA) and dissolved to 5.0 $mg \cdot mL^{-1}$ in an aqueous solution. The employed chemicals were purchased, ammonium sulphate[analytical grade (AR) from Shantou Xilong Chemical Co., (Shantou, China], potassium dihydrogen phosphate（AR）from Xi'an Chemical Co, Xi'an, China）, sodium chloride（AR）from Tianjing Jingbei Fine Chem. Co., (Tianjing, China). Acrylamide，marker of proteins (mass 14,200-97,000 Da), sodium dodecylsulpohate, trihydroxymethyl aminomethane（totally electrophoresis grade） were purchased from Sigma Co.（St. Louis, MO, USA. Coomassie brilliant blue G250 (Fluka (Mexico City, USA).

The HIC mobile phase solution A consisted of 3.0 M ammonium sulfate and 0.050 M potassium dihydrogen phosphate (pH 7.0), and solution B consisted of 0.010 M potassium dihydrogen phosphate (pH 7.0). The mobile phase of HIC with the same composition may have a varying pH in different experiments, as described in related figures. The IEC mobile phase solution C consisted of 0.02 M potassium dihydrogenphosphate (pH 6.5), and solution D consisted of 0.02 M Tris–HCl + 1.0 M sodium chloride (pH 6.5).

For the Bradford reagent, we dissolved 100 mg Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol and added 100 ml 85% (w/v) phosphoric acid. The solution was then diluted to 1 L when the dye was completely dissolved, and it was then filtered through

Whatman #1 paper just before use.

Deionized water was prepared with a Barnstead E-pure purification system (Thermo Scientific, Waltham, MA, USA).

2.3. Preparation of RNase A crude extract from bovine pancreas[69]

A 750 g slice of bovine pancreas was dried in air, and the surface fat layer was removed. This was then finely ground in a meat grinder. The ground pancreas was then placed in an aqueous solution that was double its volume with the pH adjusted to 3.8 using 1.0 M acetic acid and stirred at 4 ℃. The pH of the solution was checked at 3 h intervals and maintained at a pH of 3.5 to 4.0 by adding 1.0 M acetic acid. After 20 h, the resultant sticky mass was filtered through four layers of gauze. This extract was stored overnight at 4 ℃. After centrifugation at 18,000 rpm for 20 min, the supernatant was collected and filtered through a 4.5 μm membrane. This final extract was stored at -20 ℃ before use.

2.4 Experimental conditions for online purification of RNase A for crude extraction of bovine pancreas.[69]

Column: 2D (WCX, HIC) column (*L* × ID: 50 mm × 4.6 mm; silica-based; particle size, 5 μm; pore size, 22 nm; stainless steel tube). Mobile phase in WCX mode: solution A, 0.02 M potassium dihydrogenphosphate (pH 6.5); solution B, 0.02 M Tris–HCl + 1.0 M sodium hloride (pH 6.5). Mobile phase in HIC mode: solution C, 3.0 M ammonium sulfate + 0.02 M potassium dihydrogenphosphate (pH 6.5); solution D, 0.02 M potassium dihydrogenphosphate (pH 6.5). Elution mode: (1) separation with IEC mode: linear gradient elution used with solution B varied from 0% to 50% under 1.0 mL/min from 0 to

15 min; (2) column re-life and system re-equilibrium: isocratic elution for 100% solution C under 2.5 mL/min from 15 to 25 min; (3) buffer exchange with simultaneous re-sampling: isocratic elution for the mixture solution of 100% solution A and 100% solution C (1/2, v/v) under 3.0 mL/min from 25 to 30 min according to the operation[76]; (4) separation with HIC mode: linear gradient elution used with solution D varied from 40% to 60% under 1.0 mL/min from 30 to 45 min with a 5 min delay.

2.5 Determination of mass recovery [77]

2.5.1 Preparation for calibration of RNase A: A 0.50 mg/mL standard bovine serum albumin solution of 5, 10, 20, 30, 40, and 50 μL were separately added into plastic tubes. Then, they were diluted using a 0.15 M sodium chloride solution to 200 μL and 200 μL of the same blank mobile phase from each IEC and HIC modes as their reference solutions. Then, 2.0 mL Coomassie Brilliant Blue G-250 was added into each of these solutions and allowed to stand for 2 min. The absorbances of these solutions were measured at 595 nm. The mass recovery was calculated as:

$$\text{Recovery of mass \%} = (\text{injection mass} / \text{recovery mass}) \times 100\%$$

2.5.2 Determination of mass recovery of RNase A in the intermediate steps Two solutions of 30 μL of the 10 mg/mL standard of RNase A were injected into the employed 2D (WCX, HIC) column and separately separated with the IEC and HIC modes. The collected fractions from the two separation modes were determined according to the foregoing described procedure (Section 2.4.1).

2.5.3 Determination of total mass recovery of RNase A.

A sample solution of 30 μL of a 10 mg/mL standard of RNase A was separately

added into a coarse extract as the sample, and the other coarse extract was added as a "blank solution" and separated in parallel, as shown in Section 3.5. After five successive experiments, the mass recovery of the final purified RNase A was determined.

3. RESULTS AND DISCUSSION

3.1. Different behaviours of proteins in the steady and migration regions during nonsynchronous sampling in HIC

Fig. 1 shows the separation of six proteins and phenyl methanol. To identify the time at which the protein and small solute migration begins under gradient elution, nonsynchronous sampling was used, as reported previously. [76] Chromatogram A, shown in Fig. 1a, was acquired conventionally with synchronous sampling and gradient, whereas sampling started 3, 6, 9, 12, and 15 min after gradient elution for chromatograms B, C, D, E, and F, respectively. The retention of intact proteins using nonsynchronous sampling is the result of their different behaviours in immobilized state in the SR and in normal migration state in MR. The six chromatograms shown in Fig. 1a reveal the presence of an SR with migration starting only after a certain time. This feature is observed for proteins with both short (Cyt C and Myo, peaks 1 and 2, respectively) and long (the remaining four) retention times. The SR start time, end time, and duration are specific to each protein. This indicates that the proteins migrate significantly under gradient elution only up to a certain critical migration point (CMP). The time and displacer (water in HIC [51]) concentrations corresponding to the CMP are thus termed the critical migration time, T_{CMP}, and critical migration concentration, C_{CMP}. Thus, the protein can be considered to be stationary for

instantaneous displacer concentrations, $C_{(i,D)}$, that are lower than C_{CMP}. Additionally, as long as $C_{(i,D)} < C_{CMP}$, the resolution and retention of proteins under gradient elution are independent of the duration of the process. This indicates that the resolution and retention do not vary significantly, even though the migration concentrations are specific to each protein and if the displacer concentrations are lower than the C_{CMP}. This C_{CMP} may allow the critical mobile phase composition for synthetic polymers in PPC which is beyond the scope of this research, [42] and other reports a critical value for the protein separation in RPLC, [44]however, the said critical value in here is only a mental image that does not really exist at that time.

In addition to the delay between nonsynchronous sample injections (5 min), the same chromatographic conditions were used for the separation of benzyl methanol (Fig. 1b) as for the proteins (Fig. 1a.). However, different profiles are obtained. Under gradient elution, the retention time of benzyl methanol (29.4 min) is greater than that of RNase A (20.3 min) but with no SR.

The benzyl methanol of a small solute has a longer retention time, shows no SR on its elution curve and has a weak interaction with the stationary phase; however, the RNase A of a protein that has a shorter retention time shows the existence of SR and a strong interaction with the stationary phase.

Figure 1 here

3.2. Identification of CMP for proteins in a HIC column

The CMP corresponding to each protein may be expressed either as time, T_{CMP}, or as concentration, C_{CMP}. The retention time of the protein under synchronous sampling is

defined as T_R; T_R corresponding to i^{th} nonsynchronous sampling is given by $T_{(R,\,i)}$, and for $(i + 1)^{th}$ and $(i - 1)^{th}$ events, there is $T_{(R,\,i+1)}$ and $T_{(R,\,i-1)}$, respectively.

$T_{(R,i)}$ is plotted against T_{inj} for RNase A and for phenyl methanol in Figs. 2a and 2b, respectively. An SR is clearly observed in Fig. 2a with the curve remaining flat up to 20 min, but it is not observed in Fig. 2b in which $T_{(R,i)}$ increases for all T_{inj}. The SR is quantified as shown below.

Figure 2 here

Table 1 here

A divergence greater than three standard deviations, $\pm 3\sigma$, from the average retention time, $T_{(R,AVG)}$, that is measured over all sampling events from T_i (0.0 min) to either $T_{(i-1)}$ or $T_{(i+1)}$ (depending on which one satisfies the statistical criterion) defines significant protein migration. Thus, the protein is said to have migrated when $T_{(R,\,i)} - T_{(R,AVG)} \geq 3\sigma$ but is considered to be stationary when $T_{(R,\,i)} - T_{(R,AVG)} < 3\sigma$. The SR can therefore be defined as the duration time $T_{(R,S)}$ between when the protein arrives at the top of the chromatographic column and when the retention time reaches the 3σ threshold. The SR is defined as the critical migration time $T_{(CMP)}$. Similarly, the MR is defined as the duration $T_{(R,M)}$ between $T_{(CMP)}$ and the elution time of the protein, T_R.

The above definitions may be expressed mathematically:

In the steady region of variable 1,

$$T_{(R,S)} \leq T_{(CMP)} \tag{1}$$

and in the migration region of variable 2,

$$T_{(R,M)} = T_R - T_{(R,S)} \; (\geq T_{(CMP)}) \tag{2}$$

Consequently, significant migration should occur between $T_{(R,\,i-1)}$ and $T_{(R,i)}$.

No SR is observed in Fig. 2b, and the increase for all T_{ing} is similar to that observed on the right-hand side of the arrow shown in Fig. 2a. The SR therefore only exists in protein separation under gradient elution. The proteins also have an approximate retention time to benzyl methanol and do not precipitate in the mobile phase. The fact that proteins are stationary in the SR indicates that retention in this region does not follow plate theory. Conversely, although proteins interact with the stationary phase in the MR, they still move normally and are governed by the difference between the partition coefficients of the proteins in the two phases, i.e., by plate theory. Thus, the retention of proteins separated under gradient elution is based on these different behaviours in both of the SR and the MR of the HIC. This mechanism is only valid for protein separation under gradient elution and is not applicable to benzyl methanol here or for proteins separated under isocratic elution.

Both $T_{(R,S)}$ and $T_{(M,S)}$ are easily calculated. By taking RNase A in Fig. 1 as an example, the total retention time, t_R, was found to be the sum of the column dead time, T_0, $T_{(R,S)}$, and $T_{(R,M)}$.

$$t_R = T_0 + T_{(R,S)} + T_{(R,M)} \quad (3)$$

As shown by the arrow in Fig. 2a for RNase A, the CMP is the point where the flat curve begins to increase. The value obtained for $T_{(R,S)}$ depends not only on the nature of the protein but also on the properties of the resulting curve. For example, the higher the slope, the larger the $T_{(R,S)}$ value because the intersection between the horizontal line and 3σ will be further from the Y-axis. Mathematical modelling may be used to obtain accurate values for $T_{CMP,}$ and C_{CMP}. Here, the data were fitted to a polynomial, which for RNase A gives a $T_{(R,S)}$ and $T_{(R,M)}$ of 10.71 and 7.01 min, respectively. The values obtained via this method for all six proteins are listed in Table 1, and the results for RNase A are

highlighted in bold. The C_{CMP} values corresponding to each T_{CMP} are readily obtained.

3.3. Contributions of steady and migration regions to protein separation

Fig. 2a and Table 1 show that $T_{(R,S)}$ and T_R vary for each protein. To compare the contributions of $T_{(R,S)}$ and $T_{(R,M)}$ to T_R, the ratios $Rt_{(S/T)} = T_{(R,S)}/T_{(R)}$ and $Rt_{(M/T)} = T_{(R,M)}/T_{(R)}$ are shown as percentages in Table 1. For all proteins other than Cyt C, the contribution to the real retention time is greater from the SR than from the MR. Based on the behaviour of the proteins in these two regions, we conclude that the $T_{(R,M)}$ increases with the column length, whereas $T_{(R,S)}$ is not affected. The SR therefore occupies a relatively small proportion of the HIC column length but accounts for most of the protein retention time. In contrast, the MR occupies most of the column length but contributes less to protein retention. This explains how fast protein separation is achieved with short columns and high flow rates.

Table 2 here

Because the protein is stationary in the SR, this can be used as an operation space (OP), as highlighted in Fig. 2a, as long as the displacer concentration is maintained at $C_{water} < C_{CMP}$. Any operations, such as system equilibration, buffer exchange, pretreatment of the sample, resampling in 2D-LC, and protein folding, can be carried out online without altering the retention of the target protein. If these operations are conducted with a suitable mobile phase and under high flow rates, this will greatly accelerate the protein separation. As mentioned above, complete column regeneration and equilibration between samples is not necessary for the analysis of intact proteins, further expediting measurements. Based on various requests, different operation platforms can be established in the OP (see Section

3.5 latter).

In chromatography, the absolute retention time is an important parameter. However, the resolution, *Rs*, between the retention times of different proteins is perhaps of greater practical interest. For convenience, the peaks in the six chromatograms shown in Fig. 1 may be considered to vary symmetrically and may be considered to have similar widths. [44,8] Thus, the widths, W_A and W_B, of any pair of adjacent peaks, A and B, can be assumed to be identical. This allows the resolution of the A-B protein pair in the SR, $Rs_{(R,S)}$ and in the MR, $Rs_{(R,M)}$, to be expressed in terms of the respective spreads in the retention time, $\Delta T_{(R,S)}$ and $\Delta T_{(R,M)}$. The ratio of the resolutions in the steady and migration regions, $Rr_{(S/M)}$, is then expressed as follows:

$$Rr_{(S/M)} = Rs_{(R,S)}/Rs_{(R,M)} = \Delta T_{(R,S)}/\Delta T_{(R,M)} \quad (4)$$

The values measured for $\Delta T_{(R,S)}$, $\Delta T_{(R,M)}$, and $Rr_{(S/M)}$ for all five protein pairs are given in Table 2.

Table 1 shows that elution occurs in the order of increasing $T_{(R,S)}$, not $T_{(R,M)}$. In Table 2, $Rr_{(S/M)}$ ranges from absolute values of 0.83 to 24.17 and is negative for the two protein pairs, Lys/RNase A and α-Amy/Lys. The negative sign indicates that these two pairs of proteins show a partition coefficient of the eluted protein that is smaller for the α-Amy/Lys than for the Lys/RNase A, providing the possibility of changing the selectivity of the two pairs of proteins. Ordering these three proteins in terms of $T_{(R,S)}$ gives,

$$\text{RNase A} < \text{Lys} < \text{α-Amy} \quad (5)$$

while, in terms of $T_{(R,M)}$, the order is

$$\text{α-Amy} < \text{Lys} < \text{RNase A} \quad (6)$$

Therefore, RNase A is the first of the three proteins to leave the SR and enter the MR (after 10.71 min), but its migration is the slowest ($T_{(R,M)}$ = 7.01 min). The α-Amy is the last to leave the SR (after 19.8 min), but it has the fastest migration time (4.7 min). Thus, both the elution order and the separation resolution vary as a function of $Rr_{(S/M)}$, which means that different results will be obtained for different column lengths.

The discussion above supposes that Eq. (4) holds, (i.e., that all chromatographic peaks from the same HIC column have similar shapes and widths) suggesting that dynamic factors may be ignored. If two protein samples of identical volume are injected into two columns with different diameters, the resulting resolutions will also differ due to the dynamic effects reflected in the symmetry and sharpness of the peaks. The sample layer formed in a large-diameter cake-like bed is thinner than that formed in a smaller-diameter column. Therefore, the peaks arising from the former are more symmetrical.

These results show that the resolution and selectivity for intact protein separation can be optimized by adjusting the ratio of the retention times in the steady and migration regions ($T_{(R,S)}/T_{(R,M)}$). $T_{(R,M)}$ can be controlled by varying the column length, whereas $T_{(R,S)}$ is governed by the gradient time and slope. These parameters may be varied as required for particular protein pairs, which is in agreement with the reported results.[24] Theoretically, $T_{(R,S)}$ approaches $T_{(R,M)}$ as the column length is reduced, but this situation cannot be reproduced in practice because the stationary phase always occupies a certain volume. However, it may be possible to develop highly porous, sub-millimetre thick LC chips that would simultaneously act as both LC columns and dots, allowing ultrafast

packing and separation of multiple samples under very low pressure.

Based on this quantitative evaluation of the contributions of $T_{(R,S)}$ and $T_{(R,M)}$ to the total retention time during protein separation under gradient elution, the sample retention in HIC is shown to be governed by different sample behaviours in the SR and MR that are quite different from the conventional gradient retention factor, k^*. This factor expresses the retention character of substances under gradient elution and has been widely employed for the fast separation of small and proteins in HPLC. [43-45] However, it only corresponds to the average value of k in isocratic elution. It does not reflect the real retention value of solutes in gradient elution, and it also does not have any fractions. The summation of SR and MR has the advantage of not only being the real retention of proteins under gradient elution, but it also provides three methods of SR, or MR alone, as well as the combination of both. This provides much more information to theoretically understand the separation mechanism of proteins using gradient elution and to practically optimize the gradient mode for carrying out the "three high" request (see Section III).

The use of short columns for protein separation was demonstrated 30 years ago, [42,44,45,47,48,79-81,] but the experimental results vary and even conflict regarding whether the resolution from short columns are better, [42,44] comparable, [47-49] or worse than long columns. [46] Although the above analysis of how the short columns work is somewhat general and qualitative, it is supported by a number of theoretical investigations. [42–44,78-81]

With the presented two variables for SR and MR, the foregoing complicated circumstances can be easily explained as follows: (1) A shorter column has a better resolution than a longer one if the SR dominates protein retention; (2) A shorter column

has a worse resolution than a longer one if the MR dominates protein retention; (3) The contrasting resolution between a shorter and longer column if the SR and MR have a comparable domination.

Due to the limitations in sample loading in shorter columns, the shortest columns used over the past 30 years are still 3–5 cm long. [44,45] In a recent study on the separation of native proteins using HIC chromatographic cakes of various thicknesses (nonporous silica base, 3-μm particle size), a minimal column length (L_{min}) of 3.5–5 mm was found to be essential for acceptable protein resolution. With $L > L_{min}$, the effects of column length on the resolution were slight, but they became significant for $L < L_{min}$. [27] However, these effects were shown to result directly from the column length rather than from the associated sample loading.

3.4. One-minute separation of proteins using liquid chromatography

The phenomena discussed in the preceding section can be exploited to optimize protein separation under gradient elution. The two independent variables, $T_{(R,M)}$ and $T_{(R,S)}$, can be varied either separately or in conjunction. The former varies as a function of the partition coefficient of the proteins and depends on the column length and flow rate, while the latter is governed by the displacer concentration of the mobile phase and thus depends on the gradient slope and time. The separation of protein pairs or sets of proteins under gradient elution can thereby be selectively optimized according to their elution rate. Specifically, for rapidly eluted proteins, improvements in resolution can be achieved by changing the column length and/or the flow rate of the mobile phase. For slowly eluted

proteins, the resolution can be improved by changing the gradient slope and time. In both cases, this should lead to better chromatographic peak dispersion. Furthermore, high throughput can be achieved by performing operations (continuous sample-sample separation, 2D- or mD-LC, etc.) in the SR, and the only requirement is that the displacer concentration should be maintained below the critical level, C_{CMP}, during these operations. The optimization strategy presented here takes into account of all of these factors and is termed steady-region/migration-region optimization (SMO), considering the fact that $T_{(R,M)}$ and/or $T_{(R,S)}$ are varied.

SMO was implemented here for the ultrafast high-resolution separation of a protein mixture. To compare this approach with conventional "trial-and-error" optimization, a commercial short column (TSK gel Butyl-NPS, L × i.d.: 35 mm × 4.6 mm) was used for fast protein separation. As shown in Fig. 3, the seven proteins are separated within 5 min under linear gradient elution. However, the size of the gaps between the peaks, in particular between 0 and 1, 1 and 2 and 6 and 7, suggests that the separation time can be shortened further with no adverse effect on the resolution. Because the pressure and gradient slope in this short column were already maximal, the only way to expedite the separation while retaining a high resolution was to shorten the gradient time. Fig. 4a shows the chromatogram obtained with a 0.8 min gradient time. All other separation parameters were identical to those in Fig. 3. When we compare the area of the chromatogram shown in Fig. 3 from 1.3 to 5 min to that shown in Fig. 4a from 1.8 to 2.5 min, we observe that the resolution in Fig. 4a has become much worse than in Fig. 3. Furthermore, the process still requires 2.5 min. The gap between peaks 6 (α-Amy) and 7

(Ins) has disappeared, but spaces remain between peaks 0 (solvent and some Cyt C) and 1 (the remaining Cyt C) and between peaks 1 and 2 (Myo).

Figure 3 here

In contrast, the SMO should allow all of the spaces between the peaks to be shortened by decreasing both $T_{(R,M)}$ and $T_{(R,S)}$. Furthermore, as long as the column is longer than the threshold described above, [11] and as long as the separation is conducted with a high flow rate, rapid and high-resolution separation should be achieved.

To overcome the low sample loading that is intrinsic to conventional short columns, [44,45] a chromatographic cake was employed (L × i.d.: 4 mm × 10 mm) in which the same TSKgel Butyl-NPR was packed. The same seven proteins were separated, and besides the flow rate (which was 10 mL·min^{-1} rather than 1 mL·min^{-1}), the same chromatographic conditions were used as for the column. As shown in Fig. 4b, the resolution between Myo (2) and RNase A (3) is somewhat compromised, but for the other proteins the resolution is excellent for the first sample and over four subsequent cycles that were completed in less than 5 min.

A complete separation cycle comprises the actual separation of the seven proteins and all associated operations including the dead time between each sample. Each separation lasts 0.80 min and requires 8 mL of mobile phase, and 0.20 min and 2 mL of mobile phase are needed between each sample. This successful separation, at a resolution and speed unprecedented in LC, stems from the combined use of the SMO strategy, the chromatographic cake and the operation space. Compared with the conventional "trial-and-error" method, the advantages of the SMO strategy are clear. With smaller

packing particles, the bed volume of the chromatographic cake and the gradient elution time may be decreased even further, allowing sub-minute LC of proteins.

The sensitivity during ultrafast separation depends on the protein concentration passing through the detector cell per unit time. The concentration is governed by the competing effects of reduced gradient elution times and increased mobile phase flow rates. Taking cytochrome *C* as an example, the sensitivity differs in Fig. 4b for the chromatographic cake under a 10 $mL \cdot min^{-1}$ flow and in Fig. 3 for the short column under a 1 $mL \cdot min^{-1}$ flow due to the differences in the gradient elution times. In the different cycles in Fig. 4b, the sensitivity with the chromatographic cake can be superior, comparable or lower than for the short column. Using a smaller chromatographic cake should lead to improved sensitivity because this would lower the flow rate of the mobile phase and shorten the gradient elution time.

Figure 4 here

3.5. Fast purification of ribonuclease A (RNase A) from bovine pancreas using online 2D-LC-1C

The desired result of proteomics research is to identify possible therapeutic proteins that may be manufactured as pharmaceuticals. The purification of recombinant rsCD-14 as a native protein requires a long time and expensive, dedicated technology [71] because its intermediate requires the transfer between two offline columns, which is slow. For the protein, RNase A, sourcing from bovine pancreas is also a very complicated, time-consuming process. We performed the process using online 2D-LC, which employs a

single silica-based 2D column, as an example to validate our optimized purification methodology. [76]

According to the presented method that was illustrated in section 3.1, the critical migration concentration, C_{CMP}, of RNase A from the employed 2D column was determined to be 0.17 M sodium chloride for the IEC separation mode and 1.48 M ammonium sulfate for the HIC mode. The evaluated C_{CMP} and T_{CMP} here denote the value for the peak maximum of RNase A. For purification, however, the whole peak width must be considered. When the flow rate was 1.0 mL·min^{-1}, the experimentally determined half width, $W_{1/2}$, was 2.5 min, corresponding to 0.08 M sodium chloride for the IEC mode, and 1.5 min, corresponding to 0.05 M ammonium sulfate for the HIC mode. Further, for safety, the whole peak width, W, was arbitrarily employed to replace $W_{1/2}$ in this study. Thus, the practical safe operation limit (SOL) can be separately expressed in the form of concentration and time, C_{SOL} and T_{SOL}, as:

$$C_{SOL} = C_{CMP} \pm W \text{ (M)} \quad (7)$$

$$T_{SOL} = T_{CMP} \pm W \text{ (min)} \quad (8)$$

For two peak-pair of the IEC-HIC of RNase A here, the W for IEC will be positive sign and that for HIC will be negative sign.

In addition to the speed of purification, other criteria, such as purity, mass recovery and economy, should also be considered and optimized. In this experiment, C_{SOL} and T_{SOL} should comprehensively considered to become optimized C_{SOL} and optimized T_{SOL}, or the

conventional operation platform (OPF) should be used. All upper limits of the SOL and OPF have to be lower than their corresponding CMP values.

Fig. 5 displays the chromatogram of crude bovine pancreas extract under optimized conditions using online 2D-LC with a single 2D column (WCX, HIC), denoted 2D-(WCX/HIC)-1C. [8,9] The whole separation pathway is divided into eight regions using perpendicular dashed lines. Regions I to II represent the first IEC separation mode, regions III to IV represent the column re-life and system re-equilibration before HIC mode (during which the RNase A is not in the separation pathway but still online in the sample loop), region V is mainly for buffer exchange and re-sampling (while simultaneously removing some impure proteins that are not immobilized under these conditions), and regions VI to VIII are for HIC separation under gradient elution. The working principle of this purification is shown in Fig. 2a.

Figure 5 here

The purity and mass recovery of each intermediate step and the final product of the purified RNase, as derived from the SDS-PAGE assay shown in Fig. 6 and the Bradford method, [77] are listed in Table 3. The purity of the final RNase A was 95.8%, and the mass recovery was 93.2%. These were obtained in 45 min using only a single 2D column.

The conventional purification of native proteins or protein drugs from real samples using techniques such as offline 2D-LC, separation by precipitation, buffer exchange by dialysis, or size exclusion chromatography requires both time and energy. [71,74,75] In our developed 2D-LC-1C purification method here, the optimized C_{SOL} or T_{SOL} allow a 1.5 mL sample containing approximately 6.8 mg of total protein containing approximately 0.17

mg RNase A without pre-separation of precipitation to be loaded onto the column in 1.5 min. The separation reported here took 10 min for the IEC mode and that was less than the 15 min required for the HIC mode. Approximately 20 min was needed for accomplishing all necessary assisted operations, giving a total process time of 45 min.

Two single conventional WCX and HIC columns might also be employed to replace the single 2D (WCX, HIC) column, as long as the two parallel columns are set up on the same improved chromatograph. However, CMP, SOL, operation platform data, sample loading, and purification conditions must be measured.

We believe that the presented method could be applied to the purification of other active proteins in bovine pancreas and could also be scaled-up to preparative and productive scales. If a suitably sized chromatographic cake is employed to replace the employed short column, a much more rapid RNase A purification could be carried out. It should be noted that the online 2D-LC-1C cannot be directly employed to replace the conventional online 2D-LC MS strategy in proteomics in which two parallel columns are used. If needed, the experiment could be performed according to the method illustrated in our previous paper. [76]

Figure 6 here

Table 3 here

3.6 Strong molecular interactions between the protein HIC stationary phase in the steady region

Because proteins are dissolved in the aqueous mobile phase, the SR described in this study is similar, in terms of molecular interactions, to the bonding region in which

adhesion forces immobilize single polymer chains on solid substrates. [82]The molecular interactions are much stronger than van der Waals forces but weaker than ionic bonds. [83,84] The reason why an SR forms only for displacer concentrations below the critical value of $C_i < C_{CMP}$ still needs to be elucidated, and further studies could provide a clearer understanding of the mechanisms governing protein separation under gradient elution.

4. CONCLUSIONS

Simultaneously fast and high-resolution protein separation can be optimized under gradient elution using a combination of steady-region/migration-region optimization and a suitably sized chromatographic cake, and it can be applied to both 1D-LC and online 2D-LC. We have shown that this approach is applicable to protein pairs, complex mixtures of proteins and multiple samples, and we have also demonstrated the method via the fast purification of crude ribonuclease A extract from bovine pancreas in a closed system using online 2D-LC.

Further conclusions can be drawn from the results of this study.

1. Two variables dominating the retention of intact proteins under gradient elution are found using hydrophobic interaction chromatography (HIC), which include a steady region (SR) in which the protein is stationary, and a migration region (MR) in which the protein moves normally.

2. For most proteins, the retention, resolution and selectivity in HIC is influenced mainly by the behaviour in the SR. Short columns can be used because the retention in the SR is independent of column length. For certain proteins, however, the behaviour in the MR becomes more important, and column length effects should be taken into account.

3. The effect of the column length on the chromatographic resolution for intact proteins is a result of the competition between the behaviours in the SR and the MR. Shorter columns may improve or degrade the resolution or have no effect, depending on the target protein and the specific gradient elution conditions.

4. Rather than amounting to dead time during chromatographic runs, the SR can be used as an operation space to expedite protein separation. Buffer exchange, collection of fractions, pre-concentration of target proteins, resampling, and so on, which are typically performed offline and can take hours or even days can actually be completed in minutes or even seconds in this operation space, provided up-to-date equipment [55] and chromatographic cakes are used.

5. Using the steady-region/migration-region optimization (SMO) strategy developed here, five successive complete separations of seven standard proteins were conducted in under 5 min in a 4 mm-thick HIC chromatographic cake. Additionally, a crude ribonuclease A extract from bovine pancreas was purified in 45 min using online 2D-LC to 95.8% purity with a mass recovery of 93.2% using a single column. Thinner (sub-millimetre or even micrometre scale) chromatographic chips are proposed as the ultrafast analysis tools of the future.

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Figure Captions

Figure 1. Hydrophobic interaction chromatograms obtained for (a) six intact proteins and (b) benzyl methanol with nonsynchronous sampling. T_R: retention time for ribonuclease A; $T_{(R,M)}$: real migration time; $T_{(R,S)}$: real steady time; T_0: dead time of the column; and t_R: total retention time. For the mobile phase, solution A was composed of 0.05 M KH_2PO_4 and 3.0 M $(NH_4)_2SO_4$ (pH 7.0); solution B was composed of 0.05 M KH_2PO_4 (pH 7.0). A linear gradient elution was used with solution B and was varied from 0% to 100% from 0 to 30 min with a 10-min delay. Column L × ID: 7.5 cm × 7.5 mm. Flow rate: 1.0 mL·min^{-1}. Detection wavelength: 280 nm. Peaks: 1, cytochrome C; 2, myoglobin; 3, ribonuclease A; 4, lysozyme; 5, α-amylase; 6, insulin. Sampling delays (min): A, 0.00; B, 3.00; C, 6.00; D, 9.00; E, 12.00; F, 15

Figure 2. Retention time $T_{(R,\,i)}$ vs. sampling time T_{ing} for (a) ribonuclease A and (b) benzyl methanol. The online operation region in (a), highlighted in yellow, can be used for fast column regeneration and equilibration for 1D-LC, fast buffer exchange and pre-concentration of the collected fractions, and resampling for mD-LC. The arrow in (a) indicates the critical migration point for ribonuclease A. The data used are presented in Table 1.

Figure 3. Fast protein separation with a TSKgel Butyl-NPR column (L × i.d.: 35 mm × 4.6 mm, I.D.). Mobile phase: solution A, 3.0 M $(NH_4)_2SO_4$ + 0.01 M KH_2PO_4, pH = 6.0; solution B, 0.01 M KH_2PO_4, pH = 6.0; detection wavelength: 280 nm; linear gradient elution: 0%–100% B over 3 min; flow rate: 1.0 mL·min^{-1}. Peaks: 0, solvent; 1, cytochrome C; 2, myoglobin; 3, ribonuclease A; 4, lysozyme; 5, α-Chy; 6, α-amylase; 7, insulin.

Figure 4. Hydrophobic interaction chromatography separation of seven intact proteins using (a) a conventional column (single cycle) and (b) a chromatographic cake (five cycles). Detection wavelength: 214 nm; total protein mass: 48 μg. Conventional column (2.5-μm nonporous TSKgel Butyl-NPR, *L* × ID: 35 mm × 4.6 mm, flow rate: 1.0 mL·min^{-1}, maximum pressure: 20 MPa); HIC chromatographic cake (2.5-μm nonporous TSKgel Butyl-NPR, *L* × ID: 4 mm × 10 mm, flow rate: 10 mL·min^{-1}, maximum pressure: 16 MPa). Other experimental conditions are the same as listed in Fig. 3.

Figure 5. Chromatogram of online purification of RNase A in the crude extraction of bovine pancreas. Peak with star: RNase A. separately from IEC and HIC separation modes; perpendicular dash line: boundary of eight regions, I-VIII on the separation pathway of RNase A using online 2D-LC; dash line with arrows denotes elution modes, three parallel dash lines appear in region V; middle dash line: comprehensively optimized C_{SOL} of RNase A (2.0 M ammonium sulfate) for resampling in HIC mode; top dash line: solution C sourcing from pump B; bottom dash line: collection fraction recharging from sample loop, which was pushed by solution A from pump A.

* Sample size: 1.5 mL (0.5 mL × 3) containing 6.8 mg of total proteins and 0.17 mg of RNase A.

Figure 6. SDS-PAGE of purified RNase A of each step by online 2D-LC (WCX/HIC). Bands: 1, crude RNase A; 2, 1st D, WCX; 3, 2nd D, HIC; 4, 2D (WCX/HIC); 5, Standard RNase A; 6, Markers.

Table 1. T_{CMP}, T_R, $T_{(R,S)}$, and $T_{(R, M)}$ values of the six proteins with HIC①

Proteins	$T_{(R,S)}$(min)	T_R^*(min)②	$Rt_{(S/T)}$ ($T_{(R,S)}/T_R^*$)	$T_{(R,M)}$(min)	T_{CMP}(min)	$Rt_{(M/T)}$ ($T_{(R,M)}/T_R^*$)	$T_{(R,M),AVG}$ ±s(min)③
Cytc	4.8	9.75	49.23%	4.95	7.38	50.77%	4.95 ±0.39
Myo	9.15	13.28	64.08%	5.13	11.73	35.92%	5.13 ±0.28
RNase A	10.71	17.72	60.44%	7.01	13.29	39.56%	7.01 ±0.65
Lys	13.4	19.22	66.27%	6.82	15.98	33.73%	6.82 ±1.06
α-Amy	19.8	24.50	80.82%	4.7	22.38	19.18%	4.70 ±0.50
Ins	24.19	29.79	81.20%	5.6	26.77	18.80%	5.60 ±0.05

*The dead volume (t_0=2.58 min) was deducted. ① The data in this table corresponds to Fig. 2; ② Data here does not include dead volume; ③ Average deviation for two continuous measurements.

Table 2. Comparison of selectivity between steady and real migration times*

	$\Delta T_{(R,S)}$,min	$\Delta T_{(R,M)}$, min	$Rr_{(S/M)}{}^{*}$($\Delta T_{(R,S)}$/ $\Delta T_{(R,M)}$
Myo/Cyt-c	4.35	0.18	24.17
RNase A/Myo	1.56	1.88	0.83
Lys/RNase A	2.69	-0.19*	-14.16*
α-Amy/Lys	6.4	-2.12*	-3.02*
Ins/α-Amy	4.39	0.9	4.88

*Absolute value

Table 3 purity and mass recovery of the intermediate step and the final product of the purified RNase A

Steps(N0)	Separation modes	Purity(%)	mass recovery(%)
1	extract	2.51	100
2	1^{st}D, WCX	76.7	95.8
3	2^{nd}D, HIC	86.8	96.8
4	2D(WCX/HIC)	95.8	93.2
5	Standard RNase A	98.4	…

*, 1, crude extract; 2, first separation by IEC mode alone; 3, second separation by HIC mode alone; 4, purification of 2D(WCX/HIC) by a single 2D(WCX, HIC) column (50mm×4.6mm ID); 5, purity of standard RNase A by SDS-PAGE

Figure 1

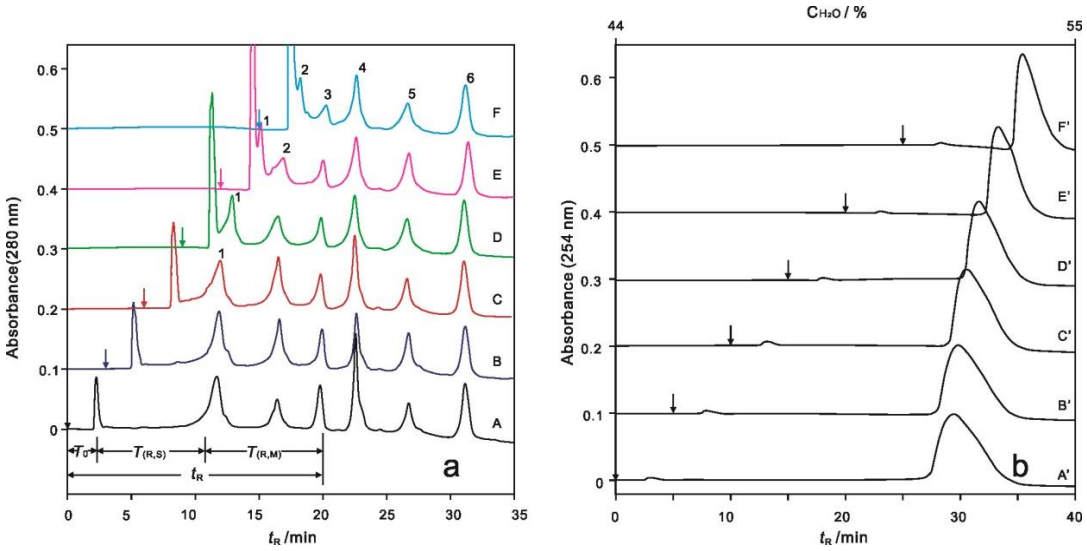

Figure 2

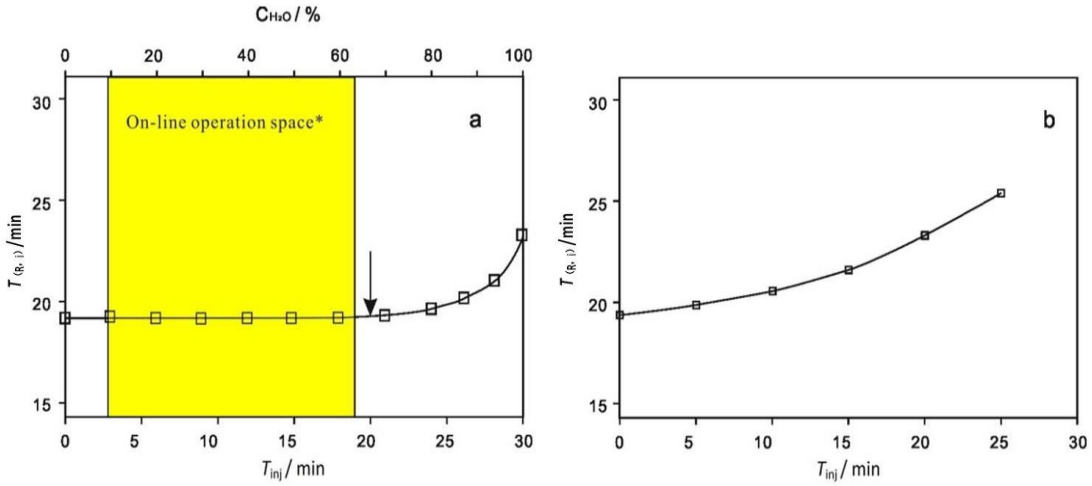

Figure 3

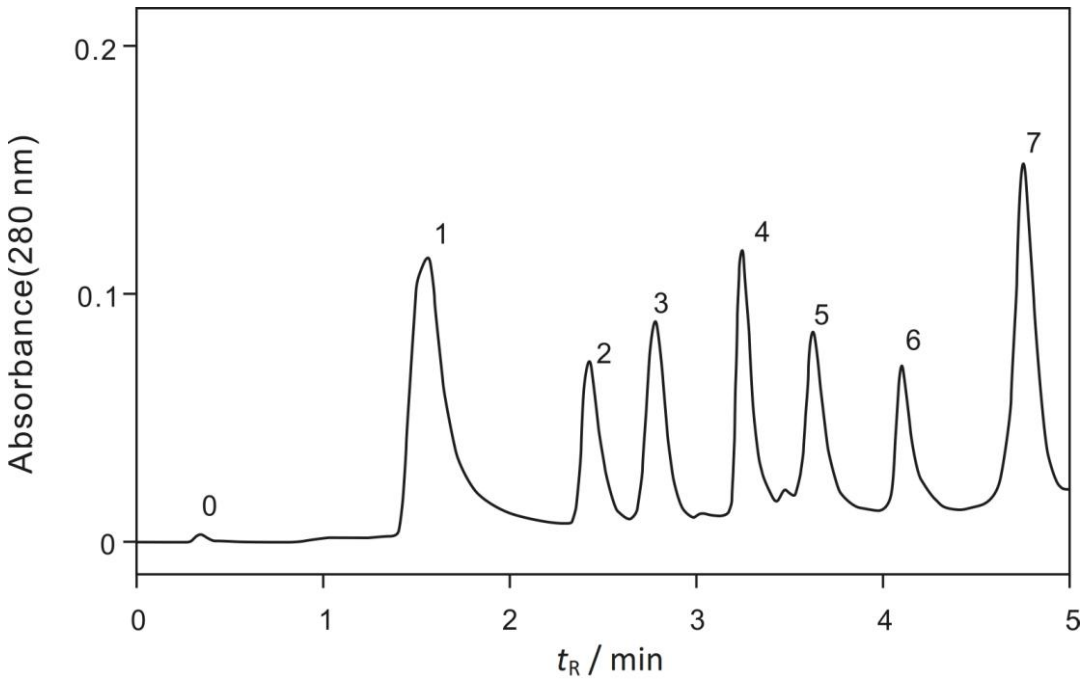

Figure 4

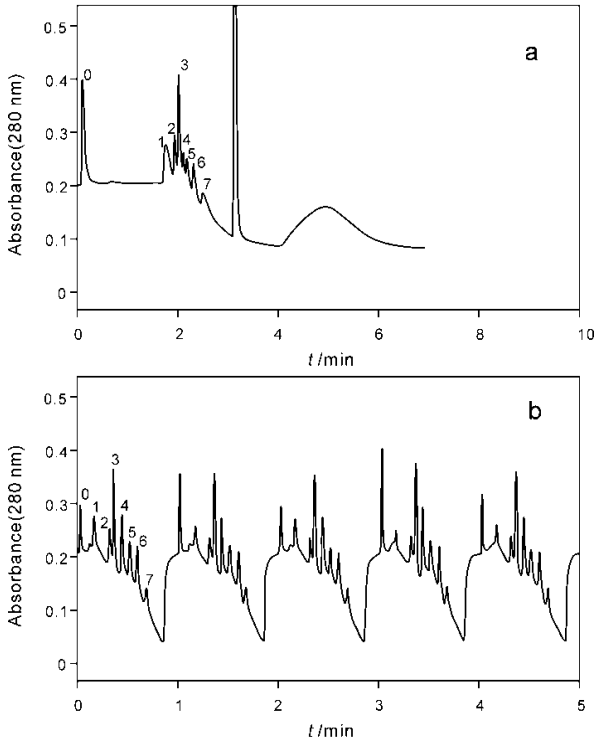

Figure 5

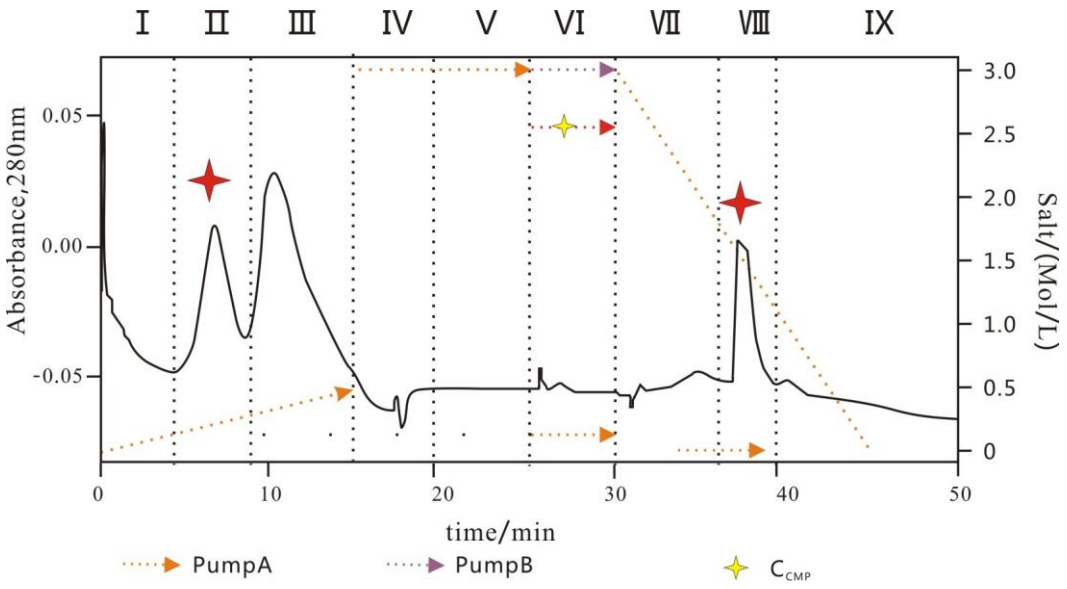

Figure 6