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# Evaluation of an embedded polar $C_4$ phase for hydrophobic protein analysis by reversed-phase liquid chromatography

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**Abstract**

A $C_4$ column (InertsilWP300 $C_4$) was developed for a protein analysis in reversed-phase chromatography. Polar groups were embedded on this phase. And this column was evaluated with low concentration of trifluoroacetic acid (TFA) and formic acid. Test $C_4$ phases that controlled carbon loading and commercially available columns were compared with this embedded $C_4$ column. The test column with higher carbon loading showed good separations of proteins at low concentrations of TFA, although hydrophobic proteins were broadened. On the other hand, embedded polar $C_4$ packings showed ideal retention behavior for proteins, which is independent of ion-pairing effects of additives in mobile phase. The reason was concluded to be the shielding effects of polar groups on the sorbents, which reduce silanophilic interactions with polar groups of proteins.


*Keywords:* Stationary phases, LC; Mobile phase composition; Proteins

## 1. Introduction

Proteins consist of several kinds of amino acids and have large molecular sizes. The structural features give undesirable adsorption that makes peak tailing and broadening in reversed-phase separations on silica-based sorbents, and it leads to unsatisfactory resolution or low recovery. It's mainly because the silanol groups are acidic and this ionic interaction results in an ion exchange retention mechanism. The problems increase with the size and/or the hydrophobicity of a protein because of the numerous interactions. Therefore, the silica-based sorbents used in reversed-phase separations of proteins demand high coverage on the surface and high inertness against polar groups.

Various types of sorbents: $C_2$ (ethyl), $C_4$ (butyl), $C_8$ (octyl), $C_{18}$ (octadecyl), cyanopropyl and phenyl groups are available for separations of proteins. It is known that long-chain phases like $C_8$ or $C_{18}$ can cause undesirable peak tailing or lower recovery of large proteins due to the high hydrophobicity. Packings with shorter alkyl chain length such as $C_4$ phase, therefore, are favorable for hydrophobic proteins, because of the faster kinetic desorption between proteins and stationary phase [1]. The effects of polar groups in stationary phase on the analysis of hydrophobic peptides were reported by Zhou and Hodges [2].

On the other hand, embedded polar phases have become popular in the past 10 years [3]. But the phases have not been investigated enough for reversed-phase chromatography of proteins, yet. The characteristics of these phases are significantly different from traditional alkyl bonded phases concerned with chemical composition and chromatographic behavior. Therefore, this phase is expected to be useful for protein applications.

In recent years, reversed-phase chromatography coupled to electrospray mass spectrometry has become a valuable tool for determination and structure analysis of peptides and proteins. Unfortunately, trifluoroacetic acid (TFA)-containing mobile phase often obstructs high sensitive determinations due to its signal suppression [4,5]. However, many applications of reversed-phase chromatography for hydrophobic proteins have been achieved in the presence of 0.1% TFA, which overcomes peak tailing and broadening.

We have developed new reversed-phase column for hydrophobic proteins. The main feature is polar groups between the $C_4$ chain and the silica surface for decreasing the contribution of silanophilic interactions.

In this study, separations of proteins are investigated with the embedded polar $C_4$ phase at low concentration of TFA or non ion-pairing additives in mobile phase.

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Table 1
Physical properties of the silica-gels used in this test for column making

| Particle size[a] (μm) | Surface area[b] ($m^2$/g) | Pore diameter[b] (Å) | Pore volume[b] (ml/g) | Metal impurity level[c] (ppm) | | | |
|---|---|---|---|---|---|---|---|
| | | | | Fe | Al | Ca | Ti |
| 5 | 150 | 300 | 1.05 | 0.9 | N.D. | 0.7 | N.D. |

[a] By laser scattering method and centrifugal sedimentation.
[b] By nitrogen adsorption method.
[c] By atomic adsorption spectrometry.

Table 2
Specification of columns used in this study

| Column | Serial number | Manufacturer | Particle size (μm) | Carbon load (%, w/w) | Bonded phase | Endcapping |
|---|---|---|---|---|---|---|
| Embedded polar $C_4$ | | GL Sciences | 5 | 3.2 | Embeded polar $C_4$ | Not endcapped |
| 300$C_4$-T001 | | | 5 | 4.3 | $C_4$ | Not endcapped |
| 300$C_4$-T002 | | | 5 | 3.0 | $C_4$ | Not endcapped |
| 300$C_4$-T003 | | | 5 | 2.3 | $C_4$ | Not endcapped |
| Zorbax 300SB-$C_3$ | USKD001444 | Agilent Technologies | 5 | 1.1 | $C_3$ | Not specified |
| Protein $C_4$ | E000518-10-1 | Vydac | 5 | Not specified | $C_4$ | Not specified |
| YMC-pack protein-RP | 041592258(W) | YMC | 5 | Not specified | Not specified | Not specified |

The column size was 150 mm × 4.6 mm i.d.

## 2. Experimental

### 2.1. Apparatus

The HPLC gradient system consisted of two PU-614 pumps, a CO-630 column oven, a UV-620 variable-wavelength UV detector, a AS-640 auto-injector, and a DMC-675 dynamic mixer (all from GL Sciences, Tokyo, Japan).

### 2.2. HPLC columns

Table 1 showed physical properties of the silica gels used in this study. The metal impurities, which play a role in enhancing the acidity of the residual silanol groups, lead to undesirable peak shape on analysis of chelating compounds (including peptides and proteins) because of irreversible interactions with hydrophilic groups of the solute [6–8].

Test columns with various carbon loading, embedded polar phase and commercially available $C_4$ columns are summarized in Table 2. For embedded polar phase, InertsilWP300 $C_4$ (GL Sciences, Tokyo, Japan) was used. This column was developed for protein analysis with 300 Å wide pore silica gel.

### 2.3. Reagents

HPLC-grade acetonitrile (Kishida, Osaka, Japan) and water (Milli-Q system, Millipore, Bedford, MA, USA) were used in the preparation of the mobile phases. Trifluoroacetic acid and Formic acid (Kishida) were used as additives to the mobile phases. Protein samples (molecular mass, isoelectric point): insulin (6000, 5.7), cytochrome c

Table 3
Comparison of protein retention times on embedded polar $C_4$ and test columns

| Number[a] | Protein | Embedded polar $C_4$ | | | 300$C_4$-T003 | | | 300$C_4$-T002 | | | 300$C_4$-T001 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0.1% TFA | 0.05% TFA | 0.02% TFA | 0.1% TFA | 0.05% TFA | 0.02% TFA | 0.1%TFA | 0.05% TFA | 0.02% TFA | 0.1% TFA | 0.05% TFA | 0.02% TFA |
| 1 | Insulin | 11.5 | 10.5 | 9.3 | 12.8 | 12.1 | 11.6 | 12.4 | 11.6 | 10.8 | 11.7 | 10.9 | 10.1 |
| 2 | Cytochrome c | 12.1 | 10.5 | 8.6 | 14.2 | 13.1 | 12.0 | 13.6 | 12.3 | 11.0 | 12.7 | 11.3 | 10.1 |
| 3 | BSA | 14.9 | 13.6 | 12.5 | 17.3 | 16.5 | 16.0 | 16.7 | 15.9 | 15.1 | 15.7 | 14.4 | 14.1 |
| 4 | Myoglobin | 17.6 | 16.1 | 14.3 | 20.9 | 19.6 | 18.3 | 20.4 | 18.9 | 17.4 | 16.1 | 16.9 | 15.6 |
| 5 | Creatine amidinohydrolase | 20.2 | 18.6 | 16.8 | 23.5 | 22.3 | 21.1 | 22.6 | 21.1 | 19.7 | 21.0 | 19.5 | 18.4 |
| 6 | Ovalbumin | 21.8 | 20.3 | 17.8 | 26.7 | 25.3 | 24.1 | 25.1 | 23.5 | 22.2 | 23.1 | 21.8 | 20.5 |
| 7 | Creatinine amidohydrolase | 23.2 | 21.9 | 18.7 | 28.7 | 27.3 | 26.2 | 27.0 | 25.6 | 24.1 | 24.2 | 22.9 | 21.8 |

The data were obtained at the same conditions as Fig. 1.
[a] Peak numbers denotes proteins shown in Fig. 1.

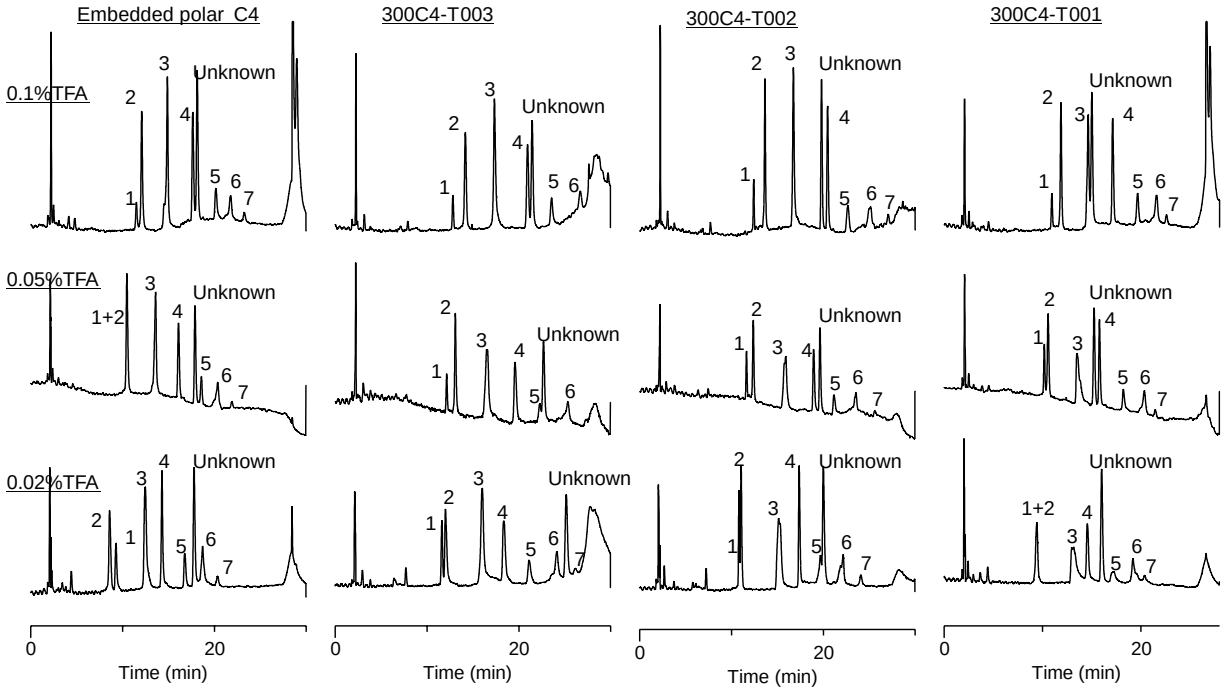


Fig. 1. Chromatograms of test mixture of proteins obtained with embedded polar $C_4$ and test columns at different TFA concentrations in mobile phase. Conditions: eluent A, water + acid; eluent B, acetonitrile–water (90:10, v/v) + acid; gradient, 20–60% B in 25 min; flow-rate, 1.0 ml/min; detection, UV at 280 nm; column temperature, 30 °C; and injected volume, 5 μl. Peaks: 1 = insulin (0.1 mg/ml), 2 = cytochrome c (0.1 mg/ml), 3 = BSA (0.2 mg/ml), 4 = myoglobin (0.2 mg/ml), 5 = creatine amidinohydrolase (0.1 mg/ml), 6 = ovalbumin (0.2 mg/ml), and 7 = creatinine amidohydrolase (0.1 mg/ml).

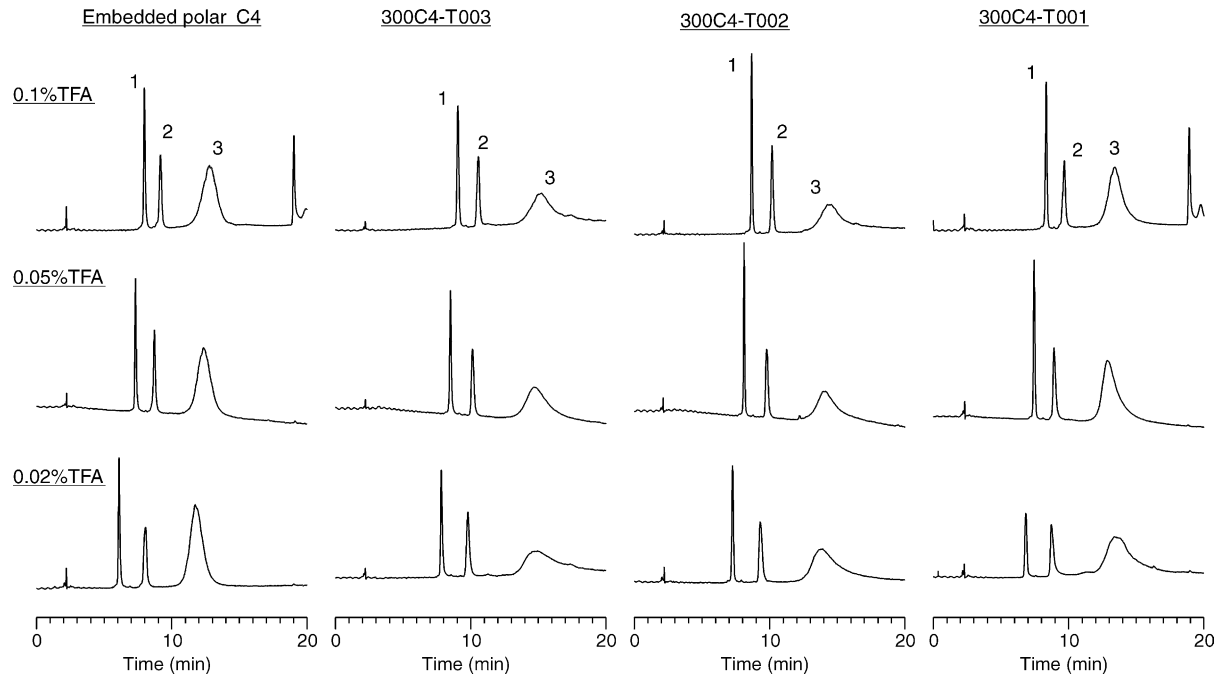


Fig. 2. Chromatograms of ferritin sample with embedded polar $C_4$ and test columns at various TFA concentrations. Conditions: eluent A, water + acid; eluent B, acetonitrile–water (90:10, v/v) + acid; gradient, 20–80% B in 20 min; flow-rate, 1.0 ml/min; detection, UV at 280 nm; column temperature, 30 °C; and injected volume, 5 μl. Peaks: 1 = cytochrome c (0.15 mg/ml), 2 = BSA (0.3 mg/ml), and 3 = ferritin (1.0 mg/ml).

Table 4
Comparison of protein retention times on embedded polar $C_4$ and commercially available columns

| Number[a] | Protein | Embedded polar $C_4$ | | | Zorbax 300SB-$C_3$ | | | Protein $C_4$ | | | YMC-pack protein-RP | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0.1% TFA | 0.05% TFA | 0.02% TFA | 0.1% TFA | 0.05% TFA | 0.02% TFA | 0.1% TFA | 0.05% TFA | 0.02% TFA | 0.1% TFA | 0.05% TFA | 0.02% TFA |
| 1 | Insulin | 11.5 | 10.5 | 9.3 | 11.5 | 10.6 | 9.7 | 11.9 | 11.5 | 12.1 | 12.8 | 12.4 | 12.9 |
| 2 | Cytochrome c | 12.1 | 10.5 | 8.6 | 13.1 | 11.6 | 10.0 | 13.6 | 12.8 | 14.0 | 13.8 | 12.5 | 12.6 |
| 3 | BSA | 14.9 | 13.6 | 12.5 | 16.3 | 15.2 | 14.4 | 17.2 | 16.5 | 17.7 | 17.1 | 15.9 | 16.6 |
| 4 | Myoglobin | 17.6 | 16.1 | 14.3 | 19.0 | 17.3 | 15.3 | 20.2 | 19.3 | 20.7 | 19.4 | 18.3 | 18.6 |
| 5 | Creatine amidinohydrolase | 20.2 | 18.6 | 16.8 | 21.6 | 20.1 | Not specified | 22.7 | 21.9 | Not specified | Not specified | Not specified | Not specified |
| 6 | Ovalbumin | 21.8 | 20.3 | 17.8 | 24.2 | 22.6 | 20.8 | 25.5 | 24.7 | 25.3 | 22.5 | 21.3 | 22.4 |
| 7 | Creatinine amidohydrolase | 23.2 | 21.9 | 18.7 | 25.7 | Not specified | Not specified | 26.9 | 26.1 | Not specified | Not specified | Not specified | Not specified |

The data were obtained at the same conditions as Fig. 4.
[a] Peak numbers denotes proteins shown in Fig. 4.

(12,000, 9.0), bovine serum albumin (BSA) (66,000, 4.7), myoglobin (17,000, 8.1), ovalbumin (45,000, 4.6), and ferritin (440,000, 4.4) were from Sigma–Aldrich (St. Louis, MO, USA). Creatine amidinohydrolase (Cdi) ($M_r$ 100,000) and creatinine amidohydrolase (Cdo) (175,000) were from Funakoshi (Tokyo, Japan).

### 2.4. Chromatographic conditions

Chromatographic conditions were described in each figure.

## 3. Results and discussion

### 3.1. Effects of TFA in mobile phase

The chromatograms of test mixture shown in Fig. 1 were obtained with embedded polar $C_4$ phase and test columns.

A phase with higher carbon loading shows longer retention of proteins, in general. However, embedded polar $C_4$ phase, which has middle carbon loading, retained proteins shorter than other normal $C_4$ phases as shown in Table 3. This is because the polar groups on the stationary phase make faster kinetic desorption of the proteins.

In the presence of 0.1% TFA, each column eluted proteins with good peak shape. At low TFA concentration, the peak shape of BSA on normal $C_4$ phases clearly showed the differences from embedded $C_4$ phase. When the concentrations of TFA were reduced to 0.05 and 0.02%, BSA was tailing on normal $C_4$ phases. The tailing of BSA was remarkably bad on the phases with lower carbon loading (300$C_4$-T001 and 300$C_4$-T002), and it seemed to be caused by the lower coverage of silanol groups. Embedded polar $C_4$ phases, which have middle carbon loading, eluted BSA and other proteins with good peak shape. It is suggested that the polar groups decrease the contribution of silanophilic interactions. Elution behaviors of hydrophobic proteins such as Cdi, ovalbumin, and Cdo on embedded polar $C_4$ phase were ideal at any conditions of TFA.

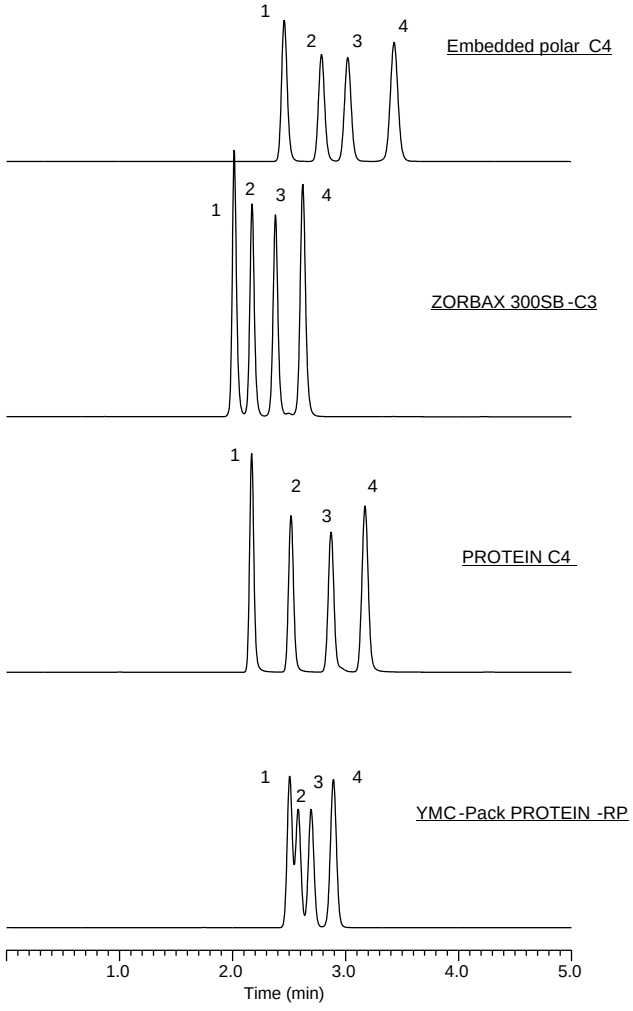


Fig. 3. Chromatograms of test mixture consisting of acetophenone, benzene, toluene, naphthalene. Conditions: eluent, acetonitrile–water (50:50, v/v); flow-rate, 1.0 ml/min; detection, UV at 254 nm; column temperature, 40 °C; and injected volume, 1 μl. Peaks: 1 = acetophenone (0.1 μl/ml), 2 = benzene (14 μl/ml), 3 = toluene (10 μl/ml), and 4 = naphthalene (1 mg/ml).

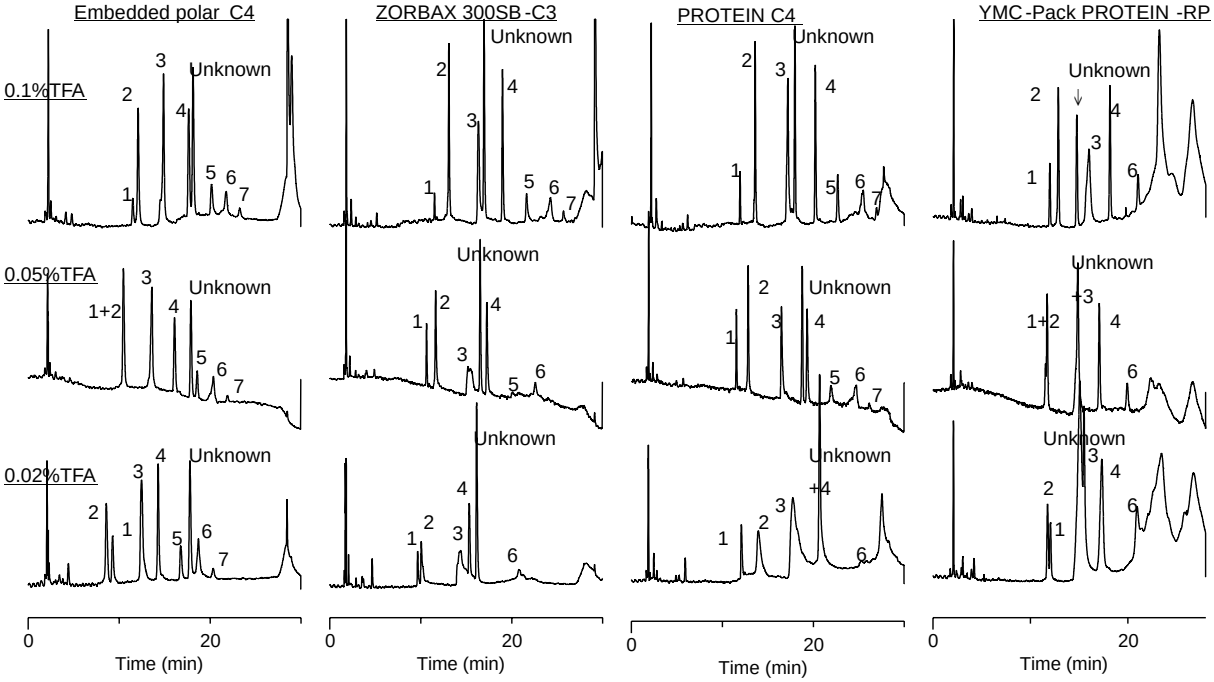


Fig. 4. Chromatograms of test mixture of proteins obtained with embedded polar $C_4$ phase and commercially available columns at various TFA concentrations. Conditions: eluent A, water + acid; eluent B, acetonitrile–water (90:10, v/v) + acid; gradient, 20–60% B in 25 min; flow-rate, 1.0 ml/min; detection, UV at 280 nm; column temperature, 30 °C; and injected volume, 5 μl. Peaks: 1 = insulin (0.1 mg/ml), 2 = cytochrome c (0.1 mg/ml), 3 = BSA (0.2 mg/ml), 4 = myoglobin (0.2 mg/ml), 5 = creatine amidinohydrolase (0.1 mg/ml), 6 = ovalbumin (0.2 mg/ml), and 7 = creatinine amidohydrolase (0.1 mg/ml).

Fig. 2 shows the chromatograms of ferritin sample separated on embedded polar $C_4$ phase and test columns. Ferritin ($M_r$ = 440,000) is one of the proteins which was not eluted with ideal behavior in the report of Burton and Snyder [9]. The size and/or the hydrophobicity of a protein have influence on undesirable adsorption because of stout interactions to stationary phase. Ferritin seemed to be suitable for evaluations of stationary phase, because the chromatograms were different between columns and strongly influenced by elution conditions.

Higher concentrations of TFA in mobile phase make hydrophobicity of proteins larger, because the protonated basic groups become uncharged by forming ion-pair with TFA. Therefore, higher carbon loading columns ($300C_4$-T002 and $300C_4$-003) could not elute ferritin with ideal peak shape as compared with $300C_4$-T001 at 0.1% TFA in mobile phase. On the other hand, lower concentration of TFA accelerated silanophilic interactions with amino groups of proteins. Therefore, the peak shape became broad at 0.02% on the low carbon loading column ($300C_4$-T001).

Embedded polar $C_4$ phase performed well at any concentration of TFA. The retention behavior of proteins was independent of the ion-pairing effects of TFA. It is suggested that the polar groups embedded on the sorbents have shielding effects, which reduce silanophilic interactions with polar groups of proteins.

### 3.2. Separation of proteins on embedded polar $C_4$ and commercially available columns

The separation of test mixture, consisting of acetophenone, benzene, toluene and naphthalene, was performed on each column for comparing their hydrophobicity in Fig. 3. Embedded polar $C_4$ phase showed longer retention of naphthalene, and it was expected that the phase has higher hydrophobicity than other commercially available columns. Proteins, however, were retained longer on commercially available columns than on embedded polar $C_4$ phase as shown in Table 4. Silanophilic interactions would contribute to the retention process of proteins on commercially available columns. At low TFA concentrations, proteins were eluted with non-ideal peak shape on commercially avail-

Table 5
Comparison of proteins recovery at 0.1% TFA and 0.2% formic acid Embedded polar $C_4$

| Protein | Recovery (%) | |
|---|---|---|
| | 0.1% TFA | 0.2% Formic acid |
| Cytochrome c | 93.1 | 92.6 |
| BSA | 96.0 | 91.0 |
| Ovalbumin | 74.1 | 80.1 |

The data were obtained at the same conditions as Fig. 4 (0.1% TFA) and Fig. 5 (0.2% formic acid).

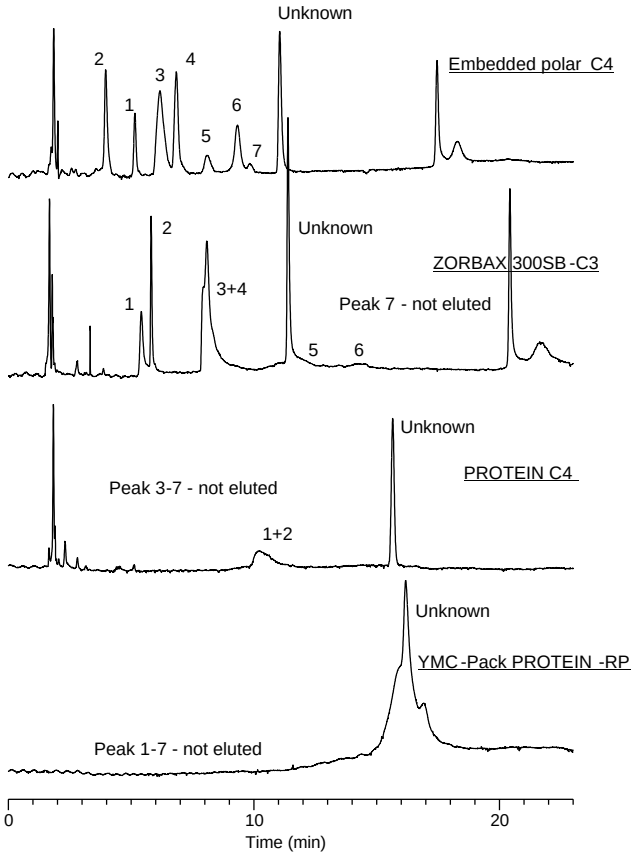


Fig. 5. Chromatograms of test mixture of proteins with embedded polar $C_4$ phase and commercially available columns at 0.2% formic acid. Conditions: eluent A, 0.2% formic acid; eluent B, acetonitrile–water (90:10, v/v) + 0.2% formic acid; gradient, 20–80% B in 20 min; flow-rate, 1.0 ml/min; detection, UV at 280 nm; column temperature, 30 °C; and injected volume, 5 μl. Peaks: 1 = insulin (0.1 mg/ml), 2 = cytochrome c (0.1 mg/ml), 3 = BSA (0.2 mg/ml), 4 = myoglobin (0.2 mg/ml), 5 = creatine amidinohydrolase (0.1 mg/ml), 6 = ovalbumin (0.2 mg/ml), and 7 = creatinine amidohydrolase (0.1 mg/ml).

able columns due to less ion-pairing effects as shown in Fig. 4.

The chromatograms of test mixture shown in Fig. 5 were obtained with embedded polar $C_4$ phase and commercially available columns in the presence of 0.2% formic acid in mobile phase. Many peaks were tailing or not eluted on commercially available columns. The test mixtures were well separated with good peak shape on embedded polar $C_4$ phase. These chromatograms demonstrated the good shielding effects of embedded polar groups, which reduce undesirable interactions on the retention process.

### *3.3. Recovery of proteins at 0.1% TFA and 0.2% formic acid*

Table 5 shows the recovery of proteins with embedded polar $C_4$ phase at the same conditions as Fig. 4 (0.1% TFA) and Fig. 5 (0.2% formic acid). Separations of cytochrome c, BSA, and myoglobin with formic acid were achieved with conventional $C_{18}$ columns by Garcia et al. [10]. Poor recoveries and resolutions of the proteins were indicated in the report. Satisfactory recoveries of cytochrome c, BSA, and ovalbumin were obtained on embedded polar $C_4$ phase both in 0.1% TFA and 0.2% formic acid. These results were led from both the shielding effects by polar groups and the faster kinetic desorption between proteins and short-chain phases.

## 4. Conclusions

Embedded polar $C_4$ phase was evaluated at low concentrations of TFA and formic acid. This phase showed good performances in elution behaviors and recoveries of proteins at 0.2% formic acid in mobile phase. Poor recovery is a problem when formic acid was used in LC–MS detection. Therefore, this phase is expected for determinations on protein analysis.

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