Effect of Acidic Additives on Peak Capacity and Detectivity in Peptide Analysis Using Nano-Flow LC/MS with Low-Density ODS Modified Monolithic Silica Capillary Columns

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Abstract

This report describes the relation between peak capacity and mass spectrometric detectivity on peptides analysis with low-density octadecylsilanated monolithic silica capillary columns for several acidic additives in mobile phase using a nano-flow LC/MS. As the acidic additives, trifluoroacetic acid (TFA), 3,3,3-trifluoropropionic acid (TriFPA), formic acid (FA), acetic acid (AA), and cyanoacetic acid (CAA) were evaluated. Peak capacity and mass spectrometric detectivity were evaluated by using the peptides mixture in the gradient elution of water/acetonitrile with 0.1% (v/v) additives at 1-50% acetonitrile composition for 35 minutes on low-density octadecyilsilated monolithic silica capillary columns. Peak capacity is usually estimated by using the ratio of gradient time to average peak width of peptides. However, we estimated the peak capacity by using the ratio of $t_0$ to the time of final peak to average peak width of peptides because the retention time of peptides was varied by difference of additives. As a result, peak capacity was increased by utilizing higher acidity additives, while the mass spectrometric detectivity was decreased. This result suggested that both high peak capacity and high detectivity for peptides analysis is irreconcilable. To overcome this relation, the impact of CAA was investigated because of its thermolysis behavior. In the condition of mobile phase with CAA at high temperature condition of mass spectrometry capillary inlet, higher peak capacity than those with TriFPA and even detectivity for those with FA were observed.

Keywords: Peak capacity; Detectivity; Acidic additives; Cyanoacetic acid; Thermolysis; Monolithic column

1. Introduction

High resolution separation and sensitive detection of highly complexed biological mixture is one of the important subjects for chromatography field. In the peptides separation, liquid chromatography (LC) combined with mass spectrometry (MS) contributes to proteomics research known as shotgun proteomics technique [1,2]. In shotgun proteomics, a reversed-phase chromatography mode with a gradient elution starting at highly aqueous condition is typically applied, because a sample matrix includes both hydrophilic/hydrophobic peptides. Furthermore, a nano-flow LC is suitable for high detectivity in MS [3,4]. Separation performance of the complexed peptides is described as peak capacity by equation 1 and 2,

$$P_c = 1 + \sqrt{\frac{N}{4} \cdot \left(\frac{(B\Delta c)^2}{(B\Delta c \cdot (t_0/t_g) + 1)}\right)} \quad (1)$$

$$\ln B = 0.6915 \ln (M_w) - 1.49 \quad (2)$$

where $P_c$: peak capacity, $N$: plate number, $B$: the slope of the plots of the natural logarithm of the retention factors against the organic solvent concentration (%), assuming 43.2 for peptide with $M_w = 2,000$, $\Delta c$: the difference in solvent composition in gradient elution, $t_0$: hold-up time, $t_g$: the gradient time, respectively [5,6]. As shown in equation 1, high peak capacity is expected by higher plate number, shallow gradient condition (large $t_g/t_0$ ratio), and wide range in mobile phase composition change.

Monolithic silica capillary columns promise to realize high peak capacity because of high plate number in practical pressure drop [7-10]. Actually, significant higher peak capacity was provided by utilizing 350 cm long ODS
monolithic silica capillary column in proteomics research [11].

For further development of monolithic silica capillary columns for peptides analysis, a control of ODS density is required. Low-density ODS modified column was suitable for highly aqueous condition for separation of polar compounds [12-14]. On the other hand, basic peptides were strongly adsorbed on the residual silanol groups without proper treatment [15].

Two approaches are considered to decrease the effect of residual silanols. One is the secondary modification of ODS silica surfaces, which is well-known as end-capping [16,17]. Another approach is addition of acidic compounds in mobile phase to prevent the interaction between acidic silanols and basic part of peptides [18]. In general, high peak capacity based on narrow peaks can be obtained for peptide separation by utilizing highly acidic additives. However, acidic additives inhibit ionization of the basic compound, resulting low detectivity in MS [19,20].

For development of the shotgun proteomics system with both high peak capacity in LC and high detectivity in MS, understanding of the effect of acidic additives for peak capacity and detectivity for low-density ODS modified columns is significantly important. In this study, we have investigated the effect of acidic additives for peak capacity and detectivity on peptides analysis with low-density ODS monolithic silica capillary columns.

2. Experimental

2.1. Chemicals

For surface modification of monolithic silica capillaries, octadecyltrimethoxysilane (ODS), 1,1,3,3,3-hexamethyldisilazane (HMDS), and 3-glycidoxypropyltrimethoxysilane (GOPTS) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Toluene (super dehydrated), methanol (super dehydrated), ethylbenzene, propylbenzene, butylbenzene and amylbenzene were purchased from Wako Pure Chemical (Osaka, Japan). For chromatographic measurements, water was purified using a Milli-Q Advantage A10 system (Merck Millipore, Darmstadt, Germany). LC/MS grade acetonitrile was purchased from Wako Pure Chemical (Osaka, Japan). Trifluoroacetic acid (TFA), 3,3,3-trifluoropropionic acid (TriFPA), formic acid (FA), acetic acid (AA) and cyanoacetic acid (CAA) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Peptide standard mixture dissolved in 0.1% formic acid aqueous solution (1.5 µg/mL, 0.75 ng) were injected to a nano-flow LC system. 0.5 µL of peptide standards (0.75 ng) were injected to a nano-flow LC system. For LC/MS measurements, peptide standard mixture dissolved in 0.1% formic acid aqueous solution (1.5 µg/mL for each peptide) was used. 0.5 µL of peptide standards (0.75 ng) were injected to a nano-flow LC system. The peptide mixture was eluted with mobile phases of water and acetonitrile including 0.1% (v/v) acidic additives in linear gradient of 1-50% acetonitrile for 35 minutes at 500 nL/min. Column temperature was kept at 40 ºC during LC separation. For the estimation of Pe, solutes were detected by a PU-2080 plus pump, a CE-2075 plus detector (JASCO, Tokyo, Japan) and a Model 7725 manual injector (IDEX Corporation, Illinois, USA) [7-10]. Chromatographic measurements for peptides separation were carried out by using Ultimate 3000 Nano LC Systems and LCQ Deca XP Plus (Thermo Fisher Scientific, Massachusetts, USA). A nano-ESI interface module was manufactured by AMR (Tokyo, Japan). A nano-electrospray was carried out with glass nano-spray emitter (I.D.; 50 µm, Tip I.D.; 15 µm, Length; 5 cm) purchased from New Objective (Massachusetts, USA).

2.2. Instruments

The surface modification reaction of monolithic silica capillaries was carried out using drying chamber purchased from AS ONE (Osaka, Japan). A split injection LC system was established for evaluation of column hydrophobicity by a PU-2080 plus pump, a CE-2075 plus detector (JASCO, Tokyo, Japan) and a Model 7725 manual injector (IDEX Corporation, Illinois, USA) [7-10]. Chromatographic measurements for peptides separation were carried out by using Ultimate 3000 Nano LC Systems and LCQ Deca XP Plus (Thermo Fisher Scientific, Massachusetts, USA). A nano-ESI interface module was manufactured by AMR (Tokyo, Japan). A nano-electrospray was carried out with glass nano-spray emitter (I.D.; 50 µm, Tip I.D.; 15 µm, Length; 5 cm) purchased from New Objective (Massachusetts, USA).

2.3. Preparation of low-density ODS modified monolithic silica capillary columns

Monolithic silica capillaries (I.D.; 100 µm, Length; 25 cm) were prepared by the method as previous reports [7-10]. Surface modification reaction of monolithic silica capillaries was carried out as follows. After filled with a dried toluene in monolithic silica capillary, ODS diluted in toluene (1.0% v/v) was poured into monolithic silica capillary, and reacted at 60 ºC for 18 hours. Then, monolithic silica capillaries were washed with toluene, acetonitrile, and methanol. Finally, remaining methoxy groups were hydrolyzed by a mixture of acetonitrile and water (70/30, v/v) containing 0.1% formic acid.

2.4. Secondary silanization of ODS monolithic silica capillary columns

Prepared ODS monolithic silica capillary columns were additionally modified with two different silanization reagents as similar method as previously described [7,8,9]. By silanization with 10% HMDS solution and GOPTS solution, additional modification of remaining silanols on ODS capillary columns completed in ODS-trimethylsilyl capillary column (ODS-TMS) and ODS-2,3-dihydroxyloxypropysilyl capillary column (ODS-DIOL), respectively.

2.5. LC and LC/MS measurement

Surface density of ODS on monolithic silica column was estimated by measurements of the ratio of retention factor for butylbenzene to amylbenzene in isocratic elution with methanol/water = 80/20 at ambient temperature.

For LC/MS measurements, peptide standard mixture dissolved in 0.1% formic acid aqueous solution (1.5 µg/mL for each peptide) was used. 0.5 µL of peptide standards (0.75 ng) were injected to a nano-flow LC system. The peptides mixture was eluted with mobile phases of water and acetonitrile including 0.1% (v/v) acidic additives in linear gradient of 1-50% acetonitrile for 35 minutes at 500 nL/min. Column temperature was kept at 40 ºC during LC separation. For the estimation of Pe, solutes were detected...
at UV214 nm on 3 nL cell (I.D.; 20 µm, Length; 10 mm). Mass spectrometric detectivity was evaluated by the intensity of peptides with the following parameters as, sheath gas flow rate; 0, aux gas flow rate; 0, spray voltage (kV); 2.0, capillary temp (ºC); 220 and 320, capillary voltage (V); 9.22, tube lens offset (V); 50, respectively.

3. Results and discussion

3.1. Estimation of the density of ODS on monolithic silica capillary columns

Fully modified ODS monolithic silica capillary columns typically show that methylene group separation factor α(CH2) at ≈1.5 [7-10]. In this case, density of ODS on silica surfaces can be estimated at 3.2 µmol/ m² [17]. Fig.1 shows the chromatogram obtained from the prepared non-endcapped ODS monolithic silica capillary column with alkylbenzenes (C6H5CnH2n+1, n =1-5) in methanol/water = 80/20 at ambient temperature. As shown in the chromatogram, α(CH2) at ≈1.4 was observed, which corresponds to ODS density around 1.5 µmol/ m² and enable to contact the aqueous mobile phase and solutes on silica surface [14].

3.2. Estimation of peak capacity for peptide mixture

Table 1 shows the properties of acidic additives utilized to evaluate the peak capacity of low-density ODS monolithic columns. It is considered that the difference of pKa value affects to the peak shape and retention behaviors of peptides.

Table 1. Property of acidic additives [18,21].

| Compound | Abbrev. | Structure | pKa |
|----------|---------|-----------|-----|
|         | TFA     | CF3 O NC | 0.43|
|         | CAA     | CF3 O OH | 2.43|
|         | TriPFA  | CF3 O OH | 3.01|
|         | FA      | OH OH CH2 O | 3.75|
|         | AA      | O OH | 4.76|

As shown in Table 1, the observed PC values were not so large. It was considered that the interaction between peptides and silanol groups was inhibited by the secondary modified diol groups, resulting both narrow peaks and small retention strength. These results indicate that the end-capping treatment for these columns was not effective to increase Pc in same gradient condition, because the end-capping contributed the decreasing of retention strength more than the narrowing of peak width.

Fig. 2 indicates the chromatograms obtained by gradient elution for peptide mixtures (table 2) utilizing TFA, TriPFA, and FA as additives for ODS-SiOH, ODS-TMS, ODS-DIOL columns. In general, estimation of peak capacity is practically carried out by using equation 3,

\[ \text{Pc}_{4\sigma} = 1 + \left[ \frac{2.35}{4} \cdot \frac{(t_g - t_0)}{w_{h,\text{ave}}} \right] \]  (3)

where, \( \text{Pc} \); obtained peak capacity for peak width at 4σ, \( t_g \); gradient time, \( t_0 \); hold-up time, \( w_{h,\text{ave}} \); average of peak width at half height, respectively.

Table 2. Property of the utilized peptides.

| Sample No. | Peptides | pKa | Mw (g/mol) |
|------------|----------|-----|------------|
| 1          | RASG-1   | 9.34| 1000.49    |
| 2          | Angiotensin frag.1-7 | 7.35| 898.47    |
| 3          | Bradykinin | 12.00| 1059.56 |
| 4          | Angiotensin II | 7.35| 1045.53 |
| 5          | Angiotensin I | 7.51| 1295.68 |
| 6          | Renin substrate | 7.61| 1757.93 |
| 7          | Enolase T35 | 7.34| 1871.96 |
| 8          | Enolase T37 | 3.97| 2827.28 |
| 9          | Melittin | 12.06| 2845.74 |

As shown in Fig. 2, the retention behaviors of peptides considerably varied by changing the columns and/or mobile phase conditions, hence it was inadequate to deal \( t_0 \) as gradient time to estimate peak capacity. Thus, we assumed \( t_0 \) as 4.5 min and \( t_g \) as the elution time of final peak (melittin) for estimation of peak capacity. Despite of narrow peaks on the ODS-DIOL column for all conditions, the observed \( \text{PCs} \) were not so large. It was considered that the interaction between peptides and silanol groups was inhibited by the secondary modified diol groups, resulting both narrow peaks and small retention strength. These results indicate that the end-capping treatment for these columns was not effective to increase \( \text{Pc} \) in same gradient condition, because the end-capping contributed the decreasing of retention strength more than the narrowing of peak width.

Fig. 3 shows the plots of obtained \( \text{Pc} \) against for pKa values of acidic additives. These plots suggest \( \text{Pc} \) depends strongly on pKa value of additives, regardless of the difference of secondary modification conditions. As a result, higher acidic additive was favorable for higher \( \text{Pc} \) separations.

3.3. Estimation of MS detectivity for peptides mixture

Effect of acidic additives for MS detectivity was
evaluated and compared with the intensity of renin substrate because relatively higher detectivity than other peptides was observed in all the conditions. Fig. 4 indicates total ion current chromatograms of peptides mixtures for 3 types of ODS monolithic columns in mobile phases containing TFA, TriPFA, and FA as additives. Obviously, the MS detectivity was affected by changing the additives. Plots of the intensity of renin substrate and $pK_a$ values were represented in Fig. 5. In contrast to the peak capacity evaluation, weak acid was favorable for high sensitive detection in MS.

3.4. A relation between peak capacity and MS detectivity

To integrate the Fig. 4 and Fig. 5, the relation between $Pc$ and MS detectivity for 3-types columns in mobile phases with TFA, TriPFA, FA, and AA was obtained by Fig.6. As shown in Fig. 6, the clear correlation between $Pc$ and MS detectivity was observed, regardless of the difference in surface modifications for low-density ODS monolithic silica columns. The result indicates that the end-capping approach was inappropriate for improving both $Pc$ and MS detectivity in low-density ODS monolithic columns.

According to the result, other acidic additive was investigated to develop the peptide analysis LC/MS system.

3.5. Impact of CAA for peak capacity and MS detectivity

As mentioned above, the clear correlation between $Pc$ and MS detectivity for low-density ODS monolithic columns was confirmed. To achieve both high $Pc$ separation and high sensitive MS detection, we investigated the CAA as an acidic additive in mobile phase.
As shown in table 1, CAA showed relatively higher acidity, $pK_a$ at $\approx 2.4$ [21]. Furthermore, CAA undergoes thermal decomposition to acetonitrile and carbon dioxide upon 160 °C. Therefore, it is expected that the removal of CAA from mobile phase at the LC/MS interface to enable high sensitive MS detection after high $P_c$ LC separation.

Fig. 4. Total ion chromatograms for 3 types of ODS columns in mobile phases containing acidic additives of (a) TFA, (b) TriPFA and (c) FA, respectively. The MS detectivity was evaluated by the intensity of renin substrate (*).

Fig. 5. Plots of the intensity of renin substrate against to $pK_a$ values of additives (TFA; circle, TriPFA; triangle, FA; square, AA; diamond) for 3 types columns.

Fig. 6. A correlation between peak capacity and MS detectivity obtained for 3-types columns for mobile phases with TFA, TriPFA, FA, and AA. Circles; TFA, triangles; TriPFA, squares; FA and diamonds; AA. Solid symbols, dotted symbols, broken symbols, represent ODS-SiOH, ODS-TMS and ODS-DIOL, respectively.

As shown in table 1, CAA showed relatively higher acidity, $pK_a$ at $\approx 2.4$ [21]. Furthermore, CAA undergoes thermal decomposition to acetonitrile and carbon dioxide upon 160 °C. Therefore, it is expected that the removal of CAA from mobile phase at the LC/MS interface to enable high sensitive MS detection after high $P_c$ LC separation. Fig. 7 represents TIC for ODS-DIOL column in mobile phases with various acidic additives. By utilizing CAA, highly retentive behavior similar to TriPFA condition and high sensitivity similar to FA condition was observed.
Fig. 8 was obtained by addition of a relation between $P_c$ and MS detectivity with CAA additive on Fig. 6. As shown in Fig. 8, a trade-off relation between $P_c$ and MS detectivity observed in typical acidic additives was overcome by utilizing CAA additive. As discussed above, a nano-flow LC/MS analysis for peptides in the condition of mobile phase with CAA additive achieved higher $P_c$ and MS detectivity than those in typical acidic additives. Therefore, it is concluded that CAA is LC/MS friendly acidic additive.

4. Conclusion

Effect of acidic additives for peak capacity and detectivity was investigated with low-density ODS monolithic capillary columns for development of peptides analysis. Higher acidity of additives in mobile phase allowed the increasing of $P_c$ and decreasing of detectivity in MS. End-capping approach apparently gave narrower peak, however, $P_c$ was not increased because of reduction of retention strength. To improve the $P_c$-detectivity relation for typical acidic additives, CAA was investigated as new additive. By utilizing CAA, both high $P_c$ and high detectivity was achieved. Therefore, a significant development in analysis of biological peptide matrix is expected by utilizing a low-density ODS monolithic column with CAA. Further investigation of low-density ODS monolithic columns and thermally degradable CAA is prospect to contribute the progress of the proteomics research.

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