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Estimation and optimization of the peak capacity of one-dimensional gradient high performance liquid chromatography using a long monolithic silica capillary column

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ABSTRACT

An estimation of the performance and optimization of gradient HPLC conditions using various columns for maximum total peak capacity was studied for "one-shot proteomics" [1] which involves one-dimensional gradient HPLC, connected to an electrospray ionization (ESI)-mass spectrometry (MS), using a monolithic silica-C_{18} capillary column (350 cm long and 100 μm internal diameter) and an over 40 h shallow gradient elution with one injection. Optimization of such special one-dimensional HPLC has been a tedious task if carried out with a trial-and-error approach due to the extremely long analysis time for each run. Here, the optimized separation conditions including the column type, either particle-packed or monolithic, and the column length with a fixed gradient time are proposed by calculating the peak capacity obtainable using a long column and a long gradient time that may promote the "one-shot proteomics" approach. For instance, conventional conditions at less than 20 MPa can be adapted for a 40 h gradient elution for the proteomics experiment, and a ca. 3 m long monolithic silica-C_{18} capillary column was identified as the optimized medium indicated by our model with peak capacity theory. To verify this model experimentally, the numbers of identified peptides and proteins were investigated with a nano LC/MS/MS system coupled with a 3 m monolithic silica-C_{18} capillary column by using various elution times. The experimental results showed that the numbers of identified peptides and proteins were maximized and reached a plateau with a gradient time of several tens of hours, which indicated that our model to optimize one dimensional HPLC conditions with a long column could be verified and useful.

1. Introduction

The separation of complex biological mixtures consisting of thousands of components remains a challenging subject for liquid chromatography. Recent advances in high-speed separations such as the use of ultra-high pressure liquid chromatography (UHPLC) [2–5] or monolithic silica columns [6,7] have considerably increased the peak capacities of one-dimensional HPLC separations as compared to more conventional HPLC methods. The next logical step could be to multiply the peak capacities of two different HPLC separations in two-dimensional HPLC (2D-HPLC), which in many cases is based on distinct physicochemical properties of the two chromatographic systems [8–10] such as a normal phase/reversed phase and an ion-exchange/reversed phase. However, an off-line 2D-HPLC system, for example, reversed-phase HPLC with a prior ion-exchange fractionation generally needs a long total analysis time and considerable human resources, as well as a lack of quantitative accuracy if the recoveries of the peptides and proteins are low. Furthermore, a complicated on-line HPLC system may induce greater chances of instrumental troubles, or involve excessive numbers of valve operations to avoid undersampling problems. In addition, the performance expected from the theoretically calculated peak capacity may not be obtained under actual experimental conditions for even proteomics research. In this sense, a simple HPLC system prior to mass spectrometric analysis is advantageous.

Monolithic silica materials offer high-efficiency separations with a long column format because of their high permeability, and have been applied to high resolution separations with a shallow gradient [7,11,12]. Currently, actual peak capacities of 350 or

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greater can be achieved when long columns and extended gradient run times are employed for proteomics research [7]. Recently, Miyamoto et al. reported that 100 μm I.D. monolithic silica capillaries of 90–1240 cm length could generate 100,000–1,000,000 theoretical plates [12]. For peptide digests, a significant improvement in resolution was obtained using a 300 cm long column under non-UHPLC conditions. Ishihama and co-workers reported the actual application of a long monolithic silica capillary of 350 cm length using a 41 h gradient, and one-dimensional LC separation under non-UHPLC conditions (<20 MPa) coupled with a MS/MS system provided the analysis of *E. coli* protein digests, which allowed the identification of 2602 proteins, which is equivalent to the number of genes detected by transcriptome analysis [1]. Their report concluded that the "one-shot" approach with a long monolithic silica capillary column and very shallow gradient elution was very promising as a front-end method for tandem mass spectrometry, and that it should be applicable to more complex organisms. Although this approach should be reliable according to previous reports, further optimization of the separation conditions, including the gradient time and column length, is necessary.

To optimize the separation conditions, the peak capacity theory, which is a well-known measure of performance for a gradient elution system, was employed in this study. Neue and co-workers reported the theory of peak capacity under standard linear gradient operating conditions for conventional reversed-phase mode chromatography, and finally described the effects of the operating conditions for the separation of large molecules such as peptides [13–15].

When a long column over a few meters in length is utilized for gradient elution, an extremely long analysis time should be necessary for optimum results based on chromatographic theory. The separation conditions, including the column length and gradient time, are often optimized retrospectively by tediously repetitive experiments. This is very time-consuming since more than dozens of hours of gradient separations are needed just to obtain one data point when optimizing the separation conditions.

Here, a method for the optimization of the separation conditions with a long gradient time is presented using the theory of peak capacity, and the expected performance of long monolithic silica capillary columns was compared with that of particle-packed columns. The method was then tested in the actual analysis of digested peptides, comparing the numbers of identified peptides and proteins.

2. Experimental

2.1. Column preparation

Tetramethoxysilane (TMOS) and methyltrimethoxysilane (MTMS) were obtained from Shinn-etsu Chemicals, and poly(ethylene glycol) (PEG, MW 10,000) from Aldrich (St. Louis, MO, USA). Water purified with a MilliQ A10 Gradient (Millipore, Bedford, MA, USA) was used in the experiments. All other chemicals and solvents were obtained from Nacalai-Tesque, Wako Pure Chemicals, Aldrich, and Tokyo Chemical Industries. The chemicals were used as obtained.

Monolithic silica capillary columns were prepared from a mixture of TMOS and MTMS (v/v 3:1 or 9:2) to form a hybrid structure. The 100 μm I.D. columns were prepared from a mixture of TMOS and MTMS as described in a previous report [16,17]. Under typical conditions, the fused-silica capillary tube was first treated with 1 M NaOH at 40 °C for 3 h, followed by a flush with water, and then kept in 1 M HCl at 40 °C for 2 h. After a flush with water and then with acetone, the capillary tube was air-dried at 40 °C. A TMOS/MTMS mixture (18 mL) was added to a solution of PEG (1.80 g) and urea (4.05 g) in 0.01 M acetic acid (40 mL) at 0 °C and stirred for 30 min. The homogeneous solution was then stirred for 10 min at 40 °C, filtered with a 0.45 μm PTFE filter, charged into a fused-silica capillary tube, and allowed to react at 40 °C. The resultant gel was subsequently aged in the capillary overnight at the same temperature. The temperature was then raised slowly (over 10–20 h for long capillary columns), and the monolithic silica columns were incubated for 4 h at 120 °C to form mesopores with the ammonia generated by the hydrolysis of urea, and then cooled and washed with methanol. After air-drying, the column was heat-treated at 330 °C for 25 h, thus causing the calcination of all organic moieties in the column. The surface modification of the silica monoliths was carried out as previously described [17–19].

2.2. Check the fundamental column performance

Capillary HPLC measurements to investigate the fundamental chromatographic performance, including the van Deemter and Kinetics plots, were performed by employing a split flow/injection HPLC system consisting of a LC-10AD vp Pump (Shimadzu, Kyoto, Japan), a 7725 injector (Rheodyne, CT, USA) with a splitting T-joint, and an on-column capillary UV detector CE-1575 (JASCO, Tokyo, Japan) or a UV detector MU701 with a capillary flow cell connected to an electronic unit with optical fibers (GL Sciences, Tokyo, Japan). A detection window (2 mm) was created by removing the polyimide coating of the fused silica capillary at a specific distance from the capillary inlet to allow on-column detection through the silica monolith for the CE-1575 detector. When on-column detection was employed, the effective column length was shorter than the total length by a few centimeters. During the experiment, the entire system, including the pump, injector, and detector, was kept at the ambient temperature. The UV chromatographic data were collected and processed by EZChrom Elite software (GL Sciences). The number of theoretical plates of a column (N) was calculated based on the peak width at half-height for symmetrical peaks.

2.3. Calculation modeling

All calculations, including the van Deemter plot, Kinetics plot, and various peak capacity plots were performed using Microsoft Excel 2010 (Microsoft, CA, USA).

2.4. Samples and materials used for the verification study

Acetonitrile, methanol, trifluoroacetic acid (TFA), acetic acid, formic acid, urea, dithiothreitol (DTT), iodoacetamide, and endoproteinase Lys-C were obtained from Wako Pure Chemical Co. (Osaka, Japan). Sequence-grade modified trypsin was obtained from Promega (Madison, WI). The water used for preparing the mobile phases was purified using a Milli-Q SP TOC (Millipore, MA, USA). The protease inhibitor cocktail was purchased from Roche Diagnostics (Basel, Switzerland). All other reagents of analytical grade were used without further treatment.

2.5. Preparation of PC-9 cell cytoplasmic protein

PC-9 cells (human lung adenocarcinoma) were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum plus antibiotics. For each experiment, one dish (5×10^7 cells/15-cm diameter dish) of confluent cells was used. Protein in PBS samples were obtained from a PC-9 cell homogenate which was prepared with a Teflon Potter-type homogenizer in the presence of a protease inhibitor cocktail, and the soluble fraction was collected as the supernatant after centrifugation with a Hitachi Himac CS150GXL (Tokyo, Japan).

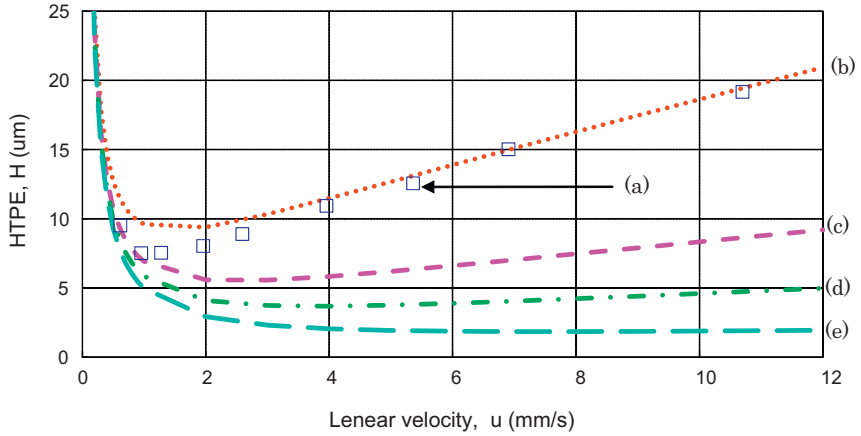

Fig. 1. van Deemter plots for various columns: (a) square plot (□): monolithic column, (b) dotted line: 5 μm particle-packed column, (c) dashed line: 3 μm particle-packed column, (d) dot-dash line: 2 μm particle-packed column, (e) long dashed line: 1 μm particle-packed column. The diffusion coefficient of a solute = 2.22×10^{-9} m^2/s is assumed for the evaluation of particle-packed columns. The described van Deemter curves were calculated for the particulate columns based on Knox equation [20], whereas measured for monolithic silica column.

2.6. Protein digestion

The amount of protein was measured using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Inc., CA, USA). The pH of the protein samples was adjusted by adding 500 mM Tris–HCl buffer (pH 8.5), and the solutions were made up to an 8 M urea final concentration. These mixtures were then reduced with DTT, alkylated with iodoacetamide, and digested with Lys-C after desalting on a PD-10 column (GE Healthcare, Buckinghamshire, UK). Next, after a 4-fold dilution with 50 mM ammonium bicarbonate, trypsin was added and the mixtures were incubated at 37 °C overnight. The digested solutions were desalted and concentrated using an Empore C18-SD disk cartridge (3 M, MN).

2.7. Verification study using LC/MS/MS

All nanoLC–MS/MS experiments were performed on an LTQ (Thermo Fisher Scientific, Bremen, Germany) connected to a Dionex Ultimate3000 nano-flow pump (Germering, Germany) and an HTC-PAL autosampler (CTC Analytics, Zwingen, Switzerland). A self-pulled capillary (10 cm length, 100 μm I.D., 6 μm opening) packed with ReproSil-Pur C18-AQ (5 μm, Dr. Maisch, Ammerbuch, Germany) was used as the C18 silica particle-packed column. A spray voltage of 2400 V was applied through a PEEK tee connector with a platinum wire. A 300 cm long monolithic silica-C18 capillary column was then prepared from a mixture of tetramethoxysilane plus methyltrimethoxylsilane to form the hybrid structure as described above. The coiled capillary column was connected to a MonoSpray FS (50 μm I.D. × 50 mm, GL Sciences, Japan) using the conductive distal coating end at which the spray voltage was applied. The flow rate was 500 nL/min. The mobile phases consisted of (A) 0.2% acetic acid and (B) 0.2% acetic acid in 80% acetonitrile. A two-step linear gradient of 5–40% B over a variable time (1–40 h), 40–100% B for 10 min, and 100% B for 20 min was employed throughout this study. The mass spectrometer was operated in data-dependent mode to automatically switch between MS full Scan and MS2. The MS scan range was m/z 300–1500, and the top precursor ions were selected from the MS scan for subsequent MS/MS scans by ion trapping. The normalized CID was set to at 35.0.

2.8. Data analysis

Mass ++ data analysis software was used to obtain a peak list, peak intensities and mass chromatograms on the basis of the recorded fragmentation spectra (available at http://groups.google.com/group/massplusplus). The peptides and proteins were identified via automated database searching using Mascot v2.1 (Matrix Science, London, UK) against the NCBInr database, with a mass tolerance of 2 Da for precursor ions, 0.8 Da for product ions and strict trypsin specificity. Carbamidomethylation was treated as a fixed modification of the cysteine residues, whereas oxidation of the methionine residues was allowed as a variable modification. One missed cleavage of trypsin was allowed. The peptides were considered to be identified if the Mascot score was over the 95% confidence limit.

3. Results and discussion

3.1. Comparison of the maximum column efficiency

Several types of columns, including a particle-packed bed and a monolithic silica capillary, were first evaluated and compared for estimating the maximum column efficiency in the 80% acetonitrile mobile phase using alkylbenzenes as solutes to establish the model foundation. Fig. 1 and Table 1 show van Deemter curves and the permeability parameters K calculated for the columns packed with several sizes of particles, 1 through 5 μm, and those obtained for the monolithic silica column. To show Fig. 1, the Knox equation [20] previously reported [12] was employed for particle-packed columns with fixed parameters, including the diffusion coefficient and flow resistance factor ϕ for the packed columns as shown below, whereas the van Deemter plots of the monolithic column were plotted by experimentally measured results without using Eq. (1):

$$h = 0.65\nu^{1/3} + \frac{2}{\nu} + 0.08\nu \quad (1)$$

Permeability parameters K shown in Table 1 was calculated with the parameters, including the linear velocity (u), viscosity of mobile

Table 1
Permeability, K values for various columns. Column porosities (ε) of 0.9 and 0.7 are assumed for monolith [30] and particle packed columns, respectively.

Particle size (μm)	K (m^2)
5	2.5×10^{-14}
3	9.0×10^{-15}
2	4.0×10^{-15}
1	1.0×10^{-15}
Monolith	9.1×10^{-14}

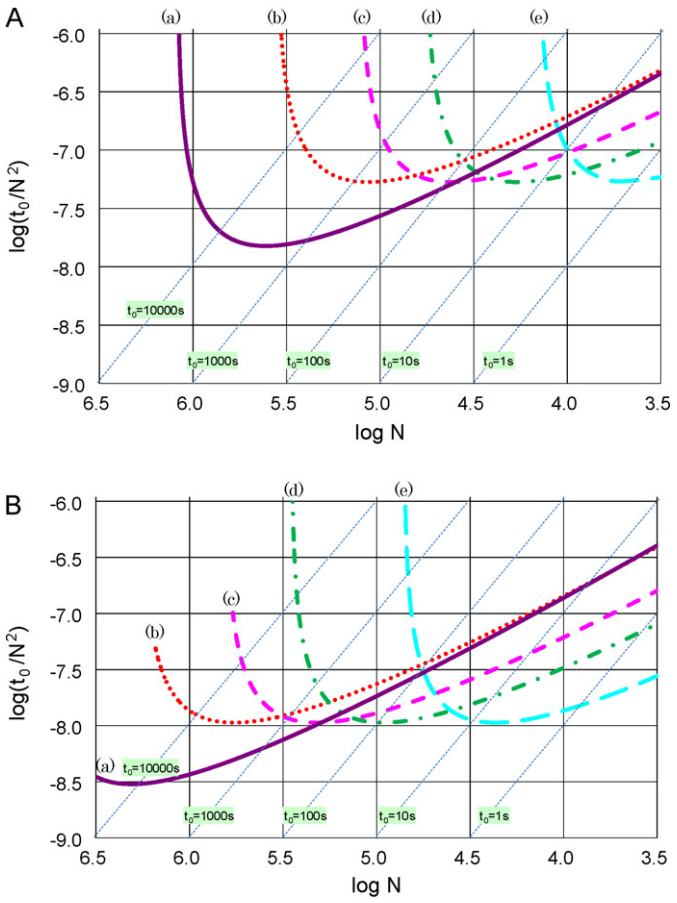

Fig. 2. Kinetics plots for various columns under changed maximum pressure. (A) Assumed maximum pressure of 20 MPa, (B) assumed maximum pressure of 100 MPa. (a) Solid line: monolithic column, (b) dotted line: 5 μm particle-packed column, (c) dashed line: 3 μm particle-packed column, (d) dot-dash line: 2 μm particle-packed column, (e) long dashed line: 1 μm particle-packed column. The diffusion coefficient of a solute = 2.22×10^{-9} m^2/s, the viscosity of mobile phase = 0.00045 Pa s [a], and the flow resistance factor ϕ = 700 were assumed for the evaluation of columns. [a]Viscosity of water–acetonitrile (v/v, 2:8) at 30 °C.

phase (η), column porosity (ε), column length (L), and pressure drop (P) as shown below [12,21]:

$$K = \frac{u\eta\varepsilon L}{P} \tag{2}$$

Fig. 1 shows that the monolithic silica column has similar column efficiency (H: theoretical plate height, u: linear velocity) as a column packed with 5 μm particles at high linear velocity. Furthermore, a higher permeability (K) was obtained, over threefold as large as a column packed with 5 μm particles as shown in Table 1. At a high linear velocity, the columns packed with small particles have advantages in H due to the reduced C term; however, high backpressure is encountered according to the K values derived by the Kozeny–Carman equation, Eq. (2) [21]. Therefore, the selection of the column type, including the particle sizes and monoliths, must be optimized according to the objectives of a study.

Fig. 2(A) and (B) shows the kinetic plots [22] of the $\log(t_0/N^2)$ values against the $\log(N)$ for the columns evaluated in Fig. 1 and Table 1, with a 20 and 100 MPa pressure limit, respectively. The dashed lines indicate the t_0 values required for the generation of a specific number of theoretical plates. The curve for the monolithic silica column is provided by Knox equation:

$$H = 3.54u^{1/3} + \frac{3.34}{u} + 1.06u$$

which was calculated by the solver function of Excel for the van Deemter plots shown in Fig. 1. The monolithic column was found to give the higher performance over the region of $4.6 < \log(N) < 6$ and $5.3 < \log(N)$ than any packed columns, even though small 1 μm particles were included under 20 and 100 MPa condition, respectively. These results indicated that 100,000 theoretical plates can be generated with a t_0 of about 250 s, 300,000 plates with a t_0 of about 1000 s, and that the present columns can theoretically give an optimum performance at around 500,000 theoretical plates with a t_0 of 3000 s under 20 MPa pressure limit. These results suggested the possibility of using the column efficiency over a range of N = 100,000–1,000,000 for even practical separations using conventional HPLC systems with pressure limit of 20 MPa as well as UHPLC systems with that of 100 MPa.

3.2. Optimization of the separation conditions by peak capacity theory

The peak capacity (PC) is a well-known measure of the efficiency of chromatographic separation systems. The PC is described by Eq. (3) for isocratic elution with t_1 and t_R giving the retention times of the first and last peaks in a chromatogram using a column showing N theoretical plates or by Eq. (4) for gradient elusions with a gradient time t_g and a peak width t_W [8,21,23–25].

$$PC = 1 + \frac{\sqrt{N}}{4} \times \ln\left(\frac{t_R}{t_1}\right) \tag{3}$$

$$PC = 1 + \frac{t_g}{t_W} \tag{4}$$

The equation for the PC for gradient elution was well reduced by Neue as shown in Eqs. (5) and (6). (B: the slope of the plots of the natural logarithm of the retention factors against the organic solvent concentration (%); Δc: the difference in solvent composition between the beginning and the end of gradient elution; t_0: the retention time of the un-retained peak; t_g: the gradient time; and N: the number of theoretical plates). Then, Eq. (7) is obtained to relate PC to the column length.

$$G = B \times \Delta c \times \frac{t_0}{t_g} \tag{5}$$

$$PC = 1 + \frac{\sqrt{N}}{4} \times \frac{B \times \Delta c}{G + 1} \tag{6}$$

$$PC = 1 + \frac{\sqrt{L/H}}{4} \times \frac{B \times \Delta c}{(B \times \Delta c \times ((L/u)/t_g) + 1)} \tag{7}$$

The value of B needed for the peak capacity calculation is not a constant, but varies according to the target molecular weight (MW). The slope B of the function $\ln k$ versus c increases with the molecular size of the peptides. Although the experimental measurement of the B values of macromolecules under isocratic elution is difficult, few reliable estimates can be found in the literature. In this work, the B values were calculated from Eq. (8) which fits well with the actual PC values described in the previous literature, which was verified to work for peptides [26].

$$\ln\ B = 0.6915\ \ln(MW) - 1.49 \tag{8}$$

One of the most interesting targets studied is digested peptides, due to the importance of proteomics research. The B value for a MW = 2000 was assumed to be ~43.2 based on extrapolations from actual research [26], and this value was well-established based on other similar estimations as well [27–29].

Fig. 3(A)–(D) shows the PC transitions in 20 MPa conventional HPLC systems upon changing the column length with various fixed gradient times, 40, 10, 1 and 0.1 h, respectively, using the conditions described above. All figures include various column types,

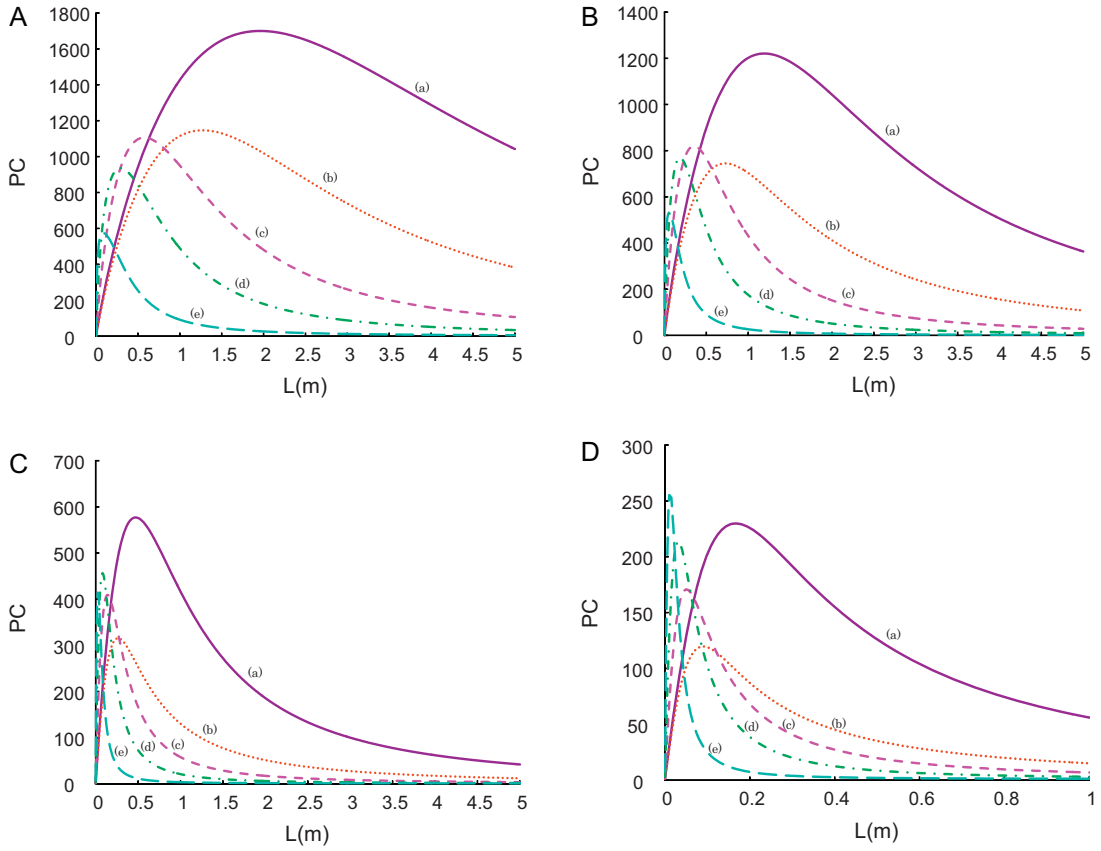

Fig. 3. PC values with various column length and types with fixed gradient time under the assumed maximum pressure of 20 MPa. (A) Gradient time: 40 h, (B) gradient time: 10 h, (C) gradient time: 1 h, (D) gradient time: 0.1 h. (a) Solid line: monolithic column, (b) dotted line: 5 μm particle-packed column, (c) dashed line: 3 μm particle-packed column, (d) dot-dash line: 2 μm particle-packed column, (e) long dashed line: 1 μm particle-packed column. Columns were evaluated with the assumed maximum pressure of 20 MPa. The diffusion coefficient of a solute = 4.5×10^{-10} m^2/s [a], the viscosity of mobile phase = 0.00101 Pa s [b], and the flow resistance factor $\phi = 700$ were assumed for the evaluation of each column. For the estimation of PC, $B = 43.2$ [26] and $\Delta c = 0.4$ were assumed. [a]Assumed diffusion coefficient of peptides [26]. [b]The highest viscosity of water–acetonitrile at 25 °C.

including monoliths, as well as columns packed with 1–5 μm particles. The maximum *PC* can be observed in all figures; for instance, the maximum *PC* values were obtained at around 2–3 m column lengths in the case of a 40 h gradient time for the monolithic silica column, and around 1–2 m column lengths in the case of a 40 h gradient time for the column packed with 5 μm particles. (Such long column lengths do not have the practical meaning for sub-2 μm particles on the current chromatograph instruments.) On the other hand, a less than 0.1 m column packed with sub-2 μm particles has the highest *PC* values when the 0.1 h gradient elution was utilized. These results suggest that a long column should need a shallow gradient time to provide the maximum *PC* values, and that monoliths or columns packed with large sized particles (e.g. over 5 μm particles) would be appropriate for optimized use for long analysis time separations. An estimation of the *PC* shows similar results as the kinetic plots shown in Fig. 2(A) and (B), as well as the method for the optimization of the separation conditions including the column types (e.g. monolith or particle-packed), column length (e.g. 0.1–3 m or more) and the optimized gradient time to provide the maximum *PC* values.

The results of the estimation under UHPLC conditions with 100 MPa pressure limit related to the use of a sub-2 μm particle-packed column are shown in Fig. 4(A)–(D), which show the *PC* transitions in UHPLC systems upon changing the column length with various fixed gradient times, 40, 10, 1 and 0.1 h, respectively. The higher *PC* values at 100 MPa conditions can be obtained in general than 20 MPa conventional conditions, and a long monolith column such as over 3 m, mainly discussed still shows the advantage for *PC* at the long time analysis, over 10 h even though the UHPLC systems are available. Meanwhile, the local maximum PC values for sub-2 mm particle-packed columns come closer to or are greater than those provided by one of the monolithic columns around 1–10 h gradient analysis under 100 MPa UHPLC conditions compared to 20 MPa conventional conditions. These plots show that the advantages of monolith column can become apparent at the use of further long column, such as over 5 m, and long gradient time like over 40 h under UHPLC systems with 100 MPa pressure limit.

3.3. Effect of the gradient time on peak capacity values

One may estimate the optimized separation conditions using a conventional HPLC system (commonly used maximum upper pressure in "omics" world: 20 MPa to prevent the instrumental troubles, such as leak from hand-tightened fittings) with a fixed column length, e.g. 3 m, which may practically be the maximum available column length. For the use of several-meter long columns under definite pressure conditions, one may hesitate to experimentally examine the optimized separation conditions, especially the "gradient time". The gradient time is strongly related to the total analysis time, and one would desire the highest *PC* in the shortest analysis time. Fig. 5(A)–(D), which were created by Eq. (7) with the different column lengths, provide the optimized gradient time to maximize the *PC*. All figures show the non-linear correlation between the *PC* values and gradient time, and a longer gradient time does not always produce higher *PC* values in a linear fashion. For instance,

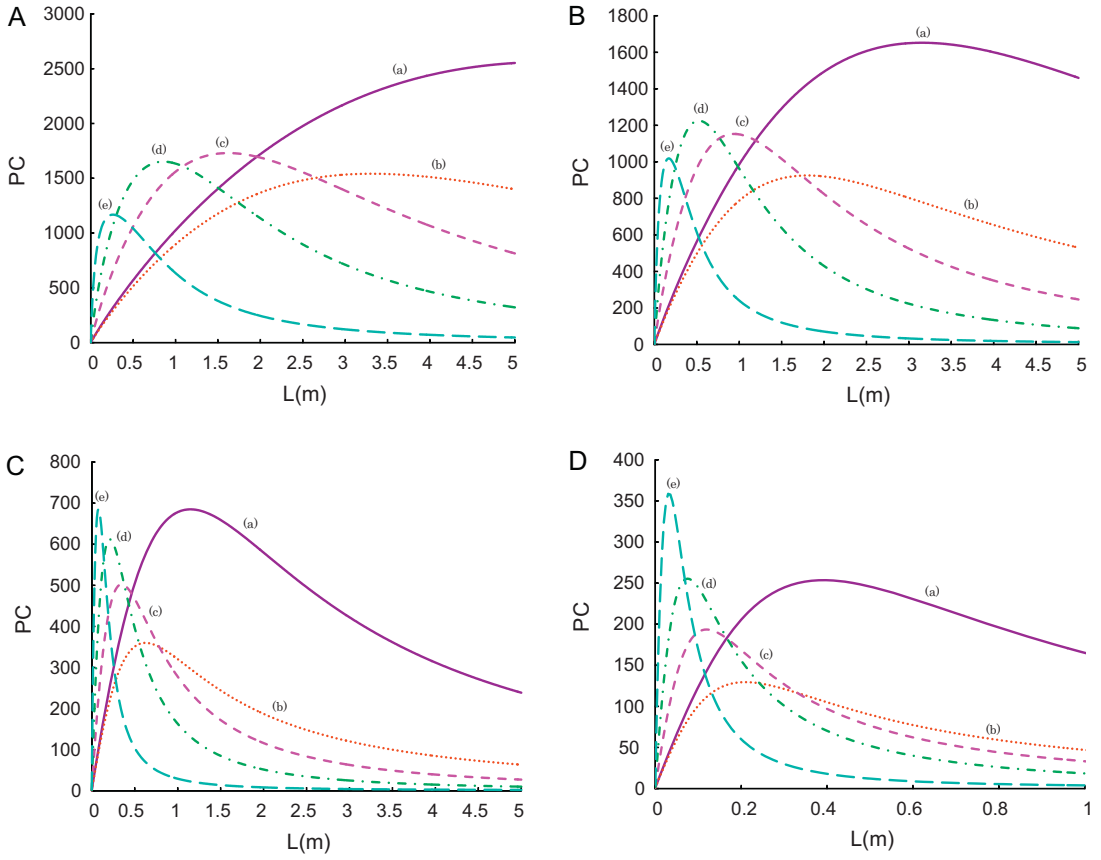

Fig. 4. PC values with various column length and types with fixed gradient time under the assumed maximum pressure of 100 MPa. (A) Gradient time: 40 h, (B) gradient time: 10 h, (C) gradient time: 1 h, (D) gradient time: 0.1 h. (a) Solid line: monolithic column, (b) dotted line: 5 μm particle-packed column, (c) dashed line: 3 μm particle-packed column, (d) dot-dash line: 2 μm particle-packed column, (e) long dashed line: 1 μm particle-packed column. Columns were evaluated with the assumed maximum pressure of 100 MPa. The other conditions for evaluation are the same as those described in Fig. 3.

a 0.1 m (10 cm) column packed with 2 μm particles provides the maximum *PC* at over 3 h of gradient elution at under 20 MPa, which was greater than any other particle size, and the *PC* value reached a plateau with a longer gradient time. Under normal circumstances, the best separation with the shortest separation time should be sought; however, should a compromise be needed, then it will most likely be realized by considering the use of Fig. 5 (A)–(D). If the 10 cm column at 20 MPa conditions is planned for use, a 2 μm packed column with an approximately 3 h elution time should be recommended for practical use, otherwise the monolith and sub-2 μm particles, including columns packed with 1 μm particles, have no utility for highly efficient separation systems operating a 10 cm column at under 20 MPa.

The results of the estimation under UHPLC conditions with 100 MPa pressure limit related to the use of a sub-2 μm particle-packed column are shown in Fig. 6(A)–(D), which show the *PC* transitions in UHPLC systems upon changing the gradient times with various fixed column length, 3, 1, 0.1 and 0.01 m, respectively. The higher *PC* values at 100 MPa conditions can be obtained in general than 20 MPa conventional conditions, and a long monolith column such as over 3 m, still shows the advantage for *PC* at any gradient time even though the UHPLC systems are available. However, the *PC* value reaches plateau at early gradient time point, around 30 h in case of the 3 m column length assumption, and the particulate column has advantages for *PC* at the use of 1 m or less column length. These plots show that the advantages of monolith column can become apparent at the use of further long column, such as over 5 m under UHPLC systems with 100 MPa pressure limit.

3.4. Optimization of the separation conditions for monolithic silica columns

Fig. 7 shows a 3D plot regarding practical examples of calculating the expected peak capacity for the gradient separation of peptides using a monolithic silica capillary column at 20 MPa. To determine the column length and gradient elution time using the monolithic silica column, this plot will be useful because the optimized conditions are clarified visually. Interestingly, the optimized separation conditions were shown to be $L = 2$–3 m length for an approximately 40 h gradient elution, and a longer column length decreases the *PC* value whereas a longer gradient time does not increase the *PC* value dramatically. These conditions were similar to those reported by Ishihama and co-workers in their retrospective optimization study.

The contour plot for this evaluation using the monolithic silica column is shown in the Supplementary Information (Figure S1). Moreover, a 3D and contour plot regarding practical examples of calculating the expected peak capacity for the gradient separation of peptides using a conventional 5 μm particle-packed column at 20 MPa are shown in Supplementary Information (Figure S2 and S3, respectively). The optimized separation conditions for 5 μm particle-packed column at 20 MPa were shown to be around $L = 1$ m length for an approximately 40 h gradient elution, and a longer column length decreases the *PC* value whereas a longer gradient time does not increase the *PC* value dramatically. In case of the typical conventional column, e.g. a column packed with 5 μm particles at $L = 10$ cm, it is estimated that no significant change of *PC* value

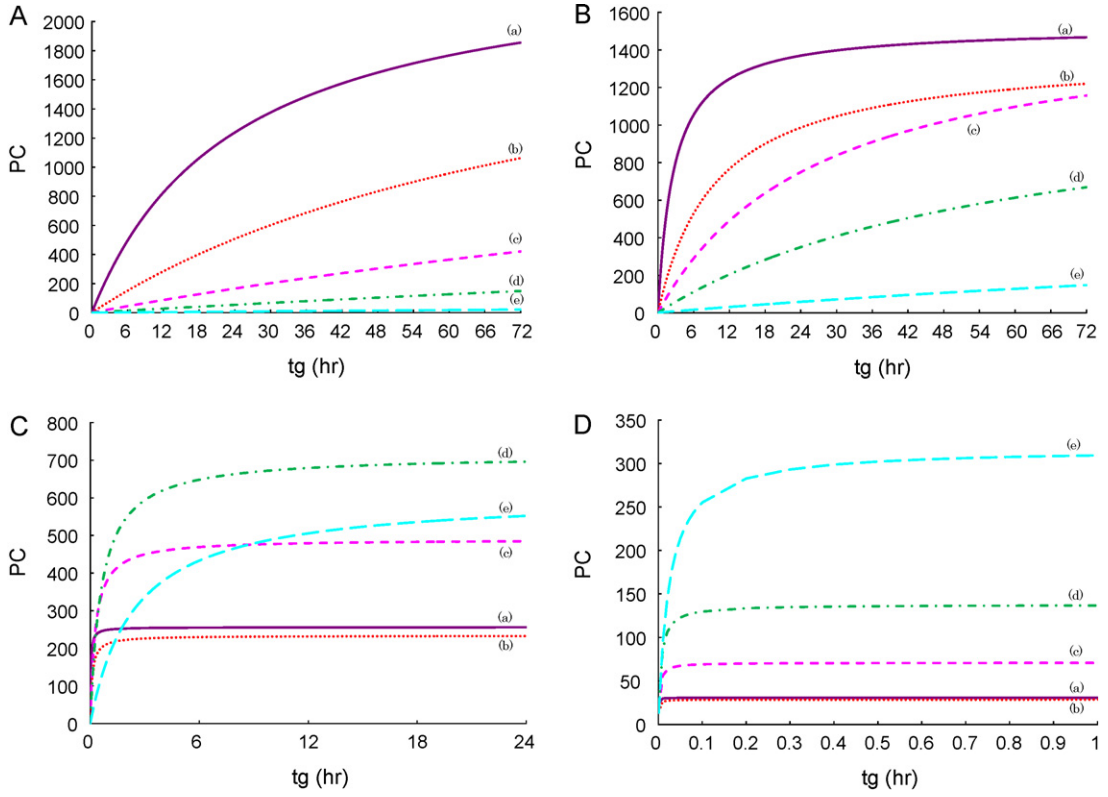

Fig. 5. PC values with various gradient time and column types with fixed column length under the assumed maximum pressure of 20 MPa. (A) Column length: 3 m, (B) column length: 1 m, (C) column length: 0.1 m, (D) column length: 0.01 m. (a) Solid line: monolithic column, (b) dotted line: 5 μm particle-packed column, (c) dashed line: 3 μm particle-packed column, (d) dot-dash line: 2 μm particle-packed column, (e) long dashed line: 1 μm particle-packed column. Columns were evaluated with the assumed maximum pressure of 20 MPa. The other conditions for evaluation are the same as those described in Fig. 3.

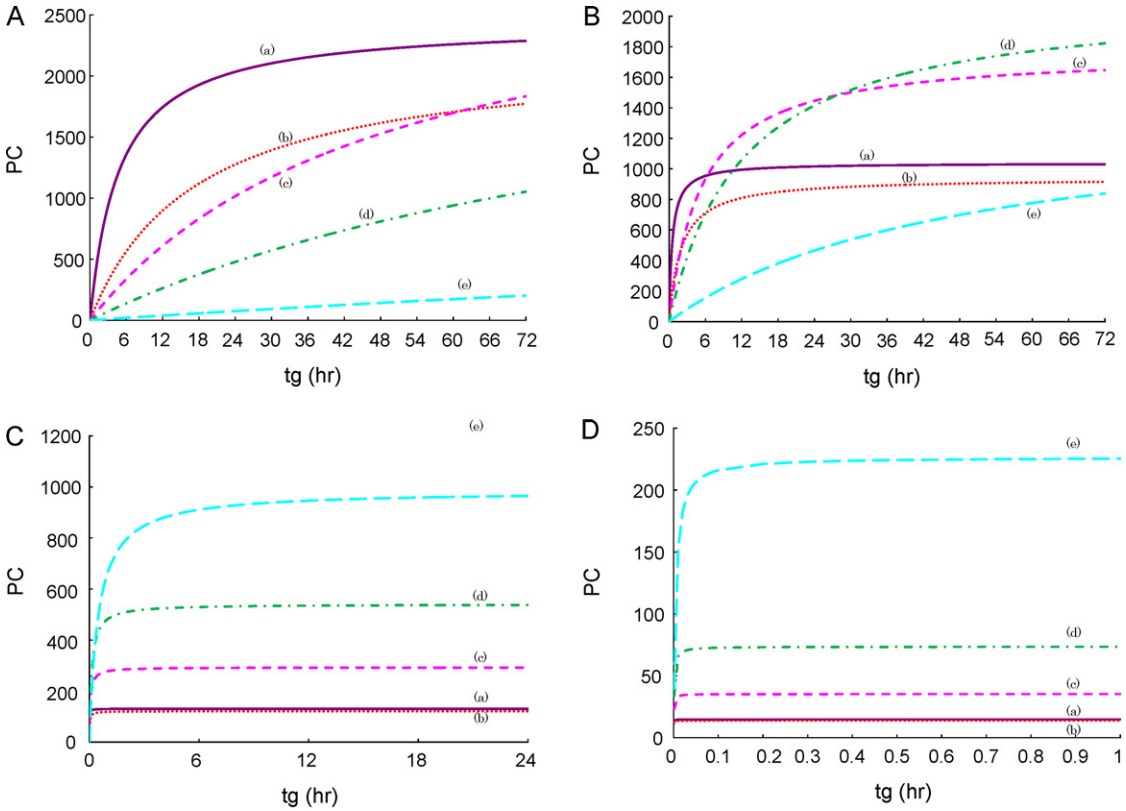

Fig. 6. PC values with various gradient time and column types with fixed column length under the assumed maximum pressure of 100 MPa. (A) Column length: 3 m, (B) column length: 1 m, (C) column length: 0.1 m, (D) column length: 0.01 m. (a) Solid line: monolithic column, (b) dotted line: 5 μm particle-packed column, (c) dashed line: 3 μm particle-packed column, (d) dot-dash line: 2 μm particle-packed column, (e) long dashed line: 1 μm particle-packed column. Columns were evaluated with the assumed maximum pressure of 100 MPa. The other conditions for evaluation are the same as those described in Fig. 3.

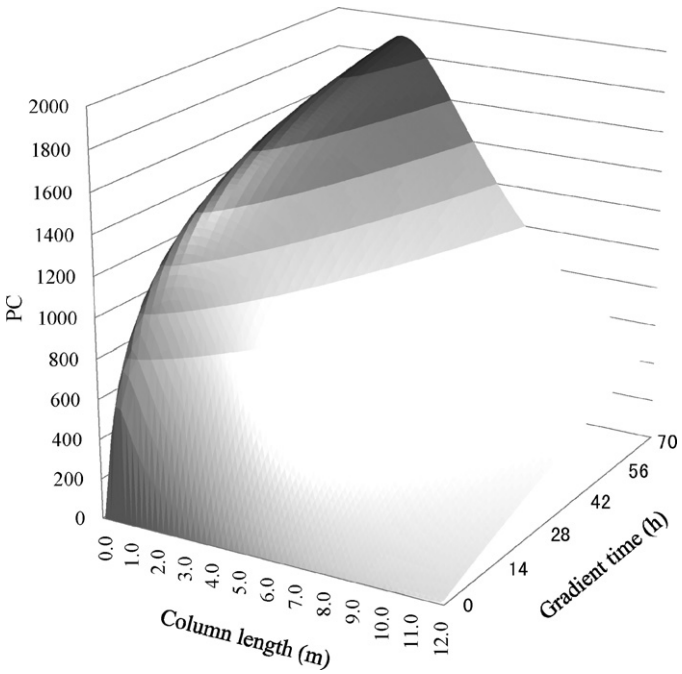

Fig. 7. 3D plot of PC values with changed column length and gradient time for monolithic silica column under the assumed maximum pressure of 20 MPa. The PC of monolithic silica column was evaluated with the assumed maximum pressure of 20 MPa. The other conditions for evaluation are the same as those described in Fig. 3.

would be observed even though a long gradient time of over 10 h is utilized.

3.5. *Verification of the estimation of the system efficiency by the peak capacity theory*

To verify the estimation of the total system performance based on the peak capacity theory as described above, the digested peptides were separated with the various gradient times and coupled with MS/MS analysis to generate the number of peptides and proteins identified. For this verification, a conventional capillary column packed with 5 μm particles (Reprosil) at $L = 10$ cm and the long monolithic silica capillary column at 3 m which was similar to the previously reported column, were employed. Using the meter long monolithic silica capillary column is an

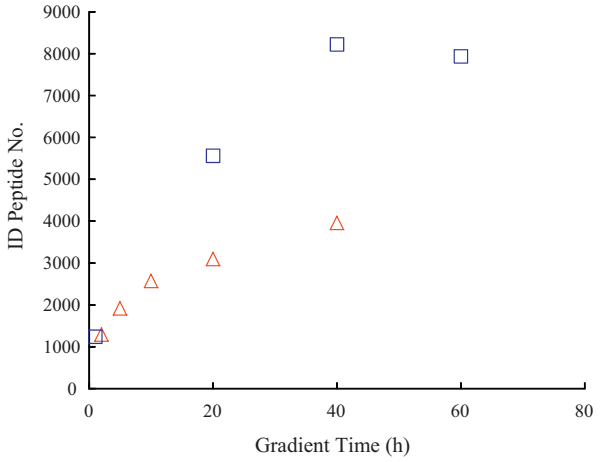

Fig. 9. Identified peptides number by various separation conditions: (a) square plot: monolithic column (100 μm I.D. × 3 m), (b) triangular plot: 5 μm particle (Reprosil)-packed column (100 μm I.D. × 10 cm). (The details for the analytical methods and identifications of peptides are shown in Section 2.)

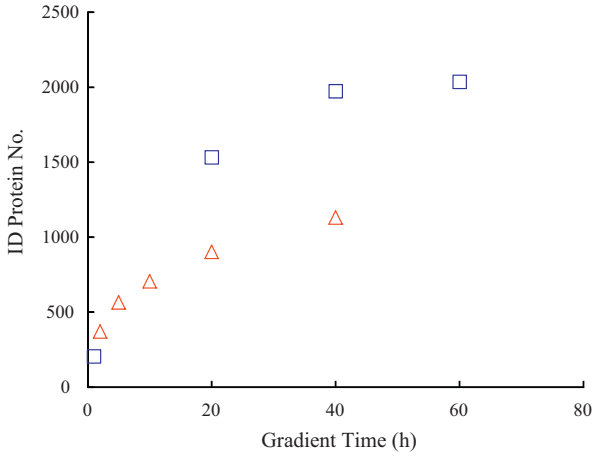

Fig. 10. Identified proteins number by various separation conditions: (a) square plot (□): monolithic column (100 μm I.D. × 3 m), (b) triangular plot (△): 5 μm particle (Reprosil)-packed column (100 μm I.D. × 10 cm). (The details for the analytical methods and identifications of proteins are shown in Section 2.)

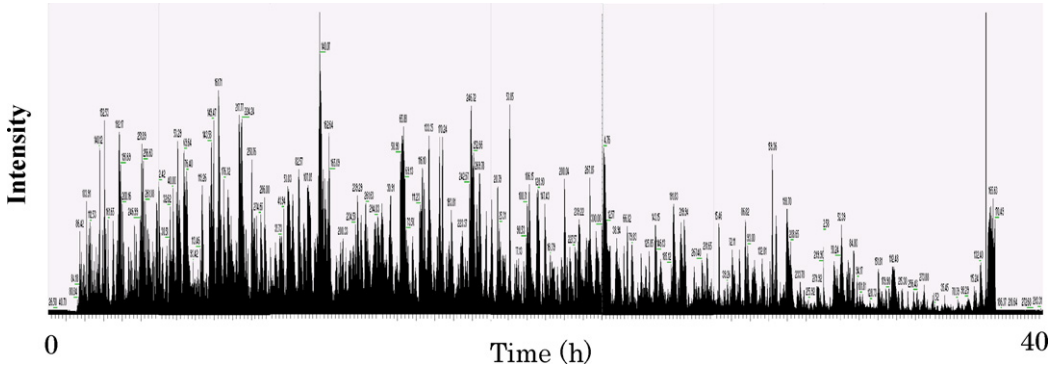

Fig. 8. Chromatogram of digested peptides by use of 3 m long monolithic silica capillary column with 40 h gradient under 20 MPa conditions. Peptides from PC-9 Cell Cytoplasmic Protein separated using a column: monolithic silica capillary column (100 μm I.D. × 3 m), stationary phase: C18 end-capped with trimethyl–silyl bond, Gradient time: 40 h, flow rate: 500 nL/min, maximum pressure: 20 MPa. The mobile phases consisted of (A) 0.2% acetic acid and (B) 0.2% acetic acid in 80% acetonitrile. A two-step linear gradient of 5–40% B over a variable time (1–40 h), 40–100% B for 10 min, and 100% B for 20 min was employed. The mass spectrometer was operated in data-dependent mode to automatically switch between MS full Scan and MS2. The MS scan range was m/z 300–1500, and the top precursor ions were selected from the MS scan for subsequent MS/MS scans by ion trapping. The normalized CID was set to at 35.0. (The details are shown in Section 2.)

interesting attempt for improving proteomics research. The optimization of the separation conditions will allow one to avoid experiments that take an extremely long analysis time and many repetitions.

Fig. 8 shows a mass-chromatogram of the digested peptides using a 3 m long monolithic silica column with a 40 h gradient time at 500 nL/min at 20 MPa conditions. (500 nL/min provides u = about 1.2 mm/s, the optimal linear velocity on van Deemter curve of the monolithic silica capillary column with 100 μm internal diameter in case of assumed porosity = 0.9.) Since the approximately 3 m monolithic silica column had the highest *PC* values with a ca. 40 h gradient separation time at 20 MPa as shown in Fig. 3(A), close to the maximum number of peptides and proteins could also be identified under these conditions. Considering that the optimized method for 3 m long monolith capillary column with a 40 h gradient time had the estimated $PC = \sim 1600$, the expected average peak widths should be around 1.5 min. Meanwhile, the range between 1.5 and 4 min were observed for the measured peak widths on mass-chromatogram, which were wider than the expected peak widths. The biases may be originated from that the analyzed samples were the digested peptides which contain many compounds with different characteristics including the molecular weight, and that induces the difficulty to lead the well-fit B value (shown in Eq. (8)) related to *PC* (or peak widths). However, the optimal HPLC conditions including column length and gradient time estimated by the mentioned method as shown in Fig. 7 are not affected by B value.

Figs. 9 and 10 show the numbers of identified peptides and proteins, respectively, under various separation conditions upon changing the gradient time for these two types of columns, including the conventional 10 cm-long capillary column packed with 5 μm particles and the long monolithic silica capillary column. As estimated from the modeling described above, a 40 h gradient elution was found to be close to optimum for the 3 m monolithic silica column in terms of the *PC*, and the numbers of identified peptides and proteins were maximized at ca. 40 h. These results support the results previously reported by Ishihama and co-workers. On the other hand, the 5 μm conventional particle-packed column provided the lower numbers of identified peptides and proteins, and more interestingly, the numbers almost reached a plateau after the 10 h gradient time as shown in Fig. 4(C), which is the simulation of the optimization of the separation conditions using a 0.1 m (10 cm) column.

In conclusion, although the absolute values of the *PC* were not directly related to the numbers of identified peptides and proteins, the results of this study implies that optimized separation conditions can be estimated by use of the peak capacity theory, and that this method may be useful for the long monolithic silica capillary columns in proteomics research. The long monolithic silica column has been used for proteomics research according to the optimal conditions estimated by the interpretation of peak capacity theory increasingly more than ever.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.12.088.

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