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Optimizing the peak capacity per unit time in one-dimensional and off-line two-dimensional liquid chromatography for the separation of complex peptide samples

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ABSTRACT

To obtain the best compromise between peak capacity and analysis time in one-dimensional and two-dimensional (2D) liquid chromatography (LC), column technology and operating conditions were optimized. The effects of gradient time, flow rate, column temperature, and column length were investigated in one-dimensional reversed-phase (RP) gradient nano-LC, with the aim of maximizing the peak per unit time for peptide separations. An off-line two-dimensional LC approach was developed using a micro-fractionation option of the autosampler, which allowed automatic fractionation of peptides after a first-dimension ion-exchange separation and re-injection of the fractions onto a second-dimension RP nano-LC column. Under the applied conditions, which included a preconcentration/desalting time of 5 min, and a column equilibration time of 12.5 min, the highest peak capacity per unit time in the 2D-LC mode was obtained when applying a short (10 min) first-dimension gradient and second-dimension RP gradients of 20 min duration. For separations requiring a maximum peak capacity of 375, one-dimensional LC was found to be superior to the off-line strong cation-exchange/×/RPLC approach in terms of analysis time. Although a peak capacity of 450 could be obtained in one-dimensional LC when applying 120-min gradients on 500-mm long columns packed with 3-μm particles, for separations requiring a peak capacity higher than 375 2D-LC experiments provide a higher peak capacity per unit time. Finally, the potential of off-line 2D-LC coupled to tandem mass spectrometry detection is demonstrated with the analysis of a tryptic digest of a mixture of nine proteins and an *Escherichia coli* digest.

1. Introduction

Determination of the proteome and identification of biomarkers are essential steps in monitoring dynamic changes in living organisms and predicting the onset of illnesses [1,2]. Different approaches are available for the identification of proteins, including shotgun proteomics. In this approach proteins are digested and the resulting peptides are separated by high-performance liquid chromatography (HPLC), followed by tandem mass-spectrometric (MS/MS) detection and identification via a database search [3]. The digestion of proteins may yield very large numbers of peptides. For example, it has been estimated that digestion of a cell lysate may produce up to 500 000 peptides with a dynamic (concentration) range of up to 10 orders of magnitude. To separate such highly complex peptide mixtures is one of the major challenges of current analytical chemistry.

Columns packed with porous micro-particulate silica stationary phases remain the gold standard for reversed-phase (RP) peptide separations with nano-LC [4,5]. Their efficacy is due to their chromatographic properties, such as high purity, outstanding mechanical strength, and large surface areas, resulting in columns with high loading capacities. The capillary format of nano-LC columns (typically 75 μm I.D.) ensures high mass sensitivity (*i.e.* signal per unit mass of analyte) – typically a factor of 5000 higher than what can be achieved with 4.6-mm I.D. columns operating at conventional flow rates [6].

To tackle contemporary proteomic samples, different strategies are followed to increase the separation performance in LC. These include the development of novel column technologies and multi-dimensional separation methods. The use of smaller particles in combination with higher column pressures to increase the peak capacity per unit time has been extensively investigated [7–11]. Long nano-LC columns have been reported to yield increased efficiencies [8,9,11,12]. Also, the development of columns with alternative morphologies, such as porous polymer monoliths, and other new stationary-phase materials for the analysis of pro-

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teins and peptides has attracted considerable attention [13–18]. LC conditions need to be adjusted to work effectively with new column formats. Wang et al. demonstrated how to tune LC conditions including gradient time, flow rate, column temperature, and eluent strength, in order to optimize peak capacity for peptide separations in reversed-phase chromatography [19]. However, it should be noted that one-dimensional (1D) LC can only be applied for relatively simple peptide mixtures.

Multi-dimensional separation approaches have the potential to separate thousands of components. To use the full peak capacity offered by two-dimensional liquid chromatography (2D-LC) the separation mechanism used in the first dimension should be orthogonal to that of the second-dimension separation [20,21]. The maximum 2D-LC peak capacity that can be obtained is the peak capacity obtained in the first dimension multiplied with that of the second-dimension separation. In MudPIT (multi-dimensional protein-identification technology) so-called "biphasic" columns are applied, often containing an ion-exchange stationary phase and an RP material in (two segments of) the same column [22]. Although the method is simple and robust, only MS compatible buffers can be used. Comprehensive on-line and, especially, off-line 2D-LC (or LC × LC) offers more flexibility in terms of separation modes and LC conditions. Since peptides can be pre-concentrated and desalted between the two dimensions, external band broadening due to the second-dimension injection volume can be minimized. This makes it possible to use a first-dimension column with a relatively large I.D. (high capacity) and a nano-LC column in the second dimension, ensuring excellent compatibility with a nano-electrospray-ionization interface for mass-spectrometric detection. This setup is easier to realize with off-line 2D-LC than when using a comprehensive 2D-LC setup. In the latter case matching eluents, flow rates, and transfer volumes is much more critical. In addition, off-line 2D-LC allows the trapping efficiency of peptides to be enhanced, since an aqueous ion-pairing solution can be added before the second-dimension separation. Moreover, off-line 2D-LC allows re-analysis of samples when partial injection of the fractions is applied. Off-line 2D-LC experiments allow a wide variety of LC conditions [23–25]. However, to the best of our knowledge no optimization strategy to obtain the best possible performance in terms of peak capacity per unit time has yet been formulated.

This report describes the optimization of the peak capacity-to-analysis time ratio for one-dimensional and off-line two-dimensional LC. The effects of gradient time, flow rate, column temperature and column length on the one-dimensional peak capacity are discussed. The effects of the first-dimension (^{1}D) ion-exchange conditions, including gradient time and sampling rate, and of the second-dimension (^{2}D) reversed-phase conditions on peak capacity and analysis time in off-line 2D-LC are discussed. Finally, the potential of 1D- and 2D-LC is demonstrated with separations of proteomic samples of varying complexity.

2. Experimental

2.1. Chemicals and materials

Acetonitrile (ACN, HPLC supra-gradient quality) and trifluoroacetic acid (TFA, ULC/MS quality) were purchased from Biosolve (Valkenswaard, The Netherlands). Sodium chloride (analytical reagent grade) and sodium dihydrogenphosphate ($\cdot 2H_2O$) were purchased from Sigma–Aldrich (Steinheim, Germany) and orthophosphoric acid (85%) from Bio Lab (Jerusalem, Israel). A digest of cytochrome *c* and a digest of a mixture of transferrin, bovine serum albumin, β-galactosidase, alcohol dehydrogenase, lysozyme, and cytochrome *c* (Dionex, Amsterdam, The Netherlands) were used for column testing. A nine-protein tryptic digest of the six proteins listed above plus β-casein, bovine serum albumin, and monoclonal antibody (mAb) and an *Escherichia coli* (*E. coli*) digest (BioRad, Paisley, UK) were used in our 2D-LC experiments.

Acclaim PepMap nano-LC columns (150 or 500 mm length; 75 μm I.D.) packed with 3-μm, 100-Å C18 particles (Dionex) and a 150 mm × 1 mm I.D. column packed with polypeptide-coated strong cation-exchange (SCX) silica particles (5 μm, 300 Å; PolyLC, Dionex) were used during the HPLC experiments. Peptide preconcentration and desalting were performed prior to a ^{2}D separation on a 5 mm × 300 μm trap column packed with Acclaim PepMap 5-μm, 100-Å C18 particles.

2.2. Instrumentation

Experiments were carried out using an UltiMate 3000 Proteomics MDLC (Dionex, Germering, Germany) equipped with a membrane degasser, ×2 dual-gradient pump system, a thermostatted Flow manager, a well-plate sampler for both injection and automated fraction collection, and two UV detectors equipped with a 45 and 3 nL Z-shaped detector flow cell, respectively. 1D-LC experiments were performed using a direct-injection setup, where the column was placed in the column compartment and connected between the injector and the UV detector with 20-μm I.D. tubing. The workflow for automated off-line 2D-LC included the following:

(1) Injection of 2–10 μL of a complex proteomic sample.
(2) A first-dimension ion-exchange separation at room temperature applying a linear salt gradient of 5 mM dihydrogenphosphate, 1 M sodium chloride solution (pH = 3), detection at 214 nm, and automated fraction collection. The sampling time (*i.e.* the time for collecting one fraction) was adjusted to the sample complexity. Detailed gradient conditions are provided in figure captions.
(3) 5 μL of a solution containing 0.1% TFA in water:acetonitrile (99:1) was added and mixed by the autosampler (20 μL was aspirated and dispensed twice) to enhance trapping efficiency.
(4) Repeated cycles of injection, 5-min preconcentration and desalting at a flow of 40 μL/min, and a second-dimension reversed-phase separations of all the fractions. Detection with UV at 214 nm. MS/MS data were generated with an ion-trap mass spectrometer (HCTultra, Bruker, Bremen, Germany) coupled on-line to the LC system via an ESI source.

The mass spectrometer was operated in the positive ionization mode in the scan range from m/z 300–1600, using a dry-gas stream of 4 L/min, nebulizer gas at 138 kPa, drying temperature of 300 °C, and a target mass set to m/z = 800. Proteins were identified through a peptide search using the MASCOT software package (Matrix Science, London, UK).

3. Results and discussion

3.1. Optimizing peak capacity for reversed-phase peptide separations

Peptide-mapping experiments are typically performed on reversed-phase nano-LC columns in gradient-elution mode, due to the excellent compatibility with mass-spectrometric detection via electrospray interfacing. A good performance criterion for the gradient separation of peptides is peak capacity (n_c) which is defined as the maximum number of peaks that can be separated with a resolution of 1 and elute in the applied gradient window. In the simplest form this peak capacity is equal to:

$$n_c \approx \frac{t_G}{W} + 1 \quad (1)$$

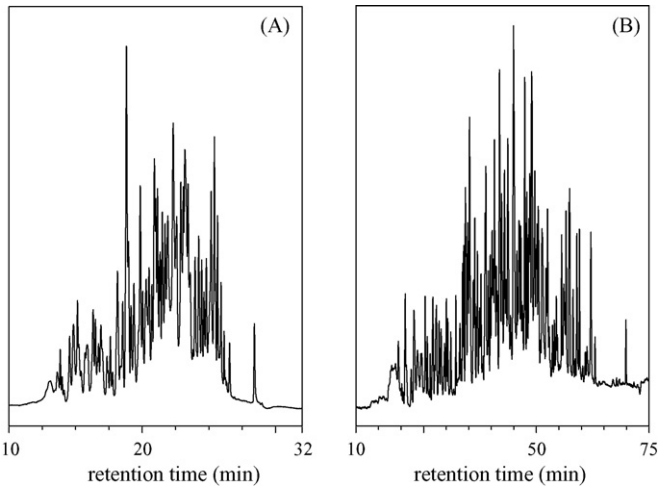

Fig. 1. Separation of a digest of a mixture of six proteins on a 15-cm column packed with 3-μm C18 particles using a gradient time of 15 min (A) and 60 min (B). Other LC conditions: flow rate, 300 nL/min; mobile phase A: 0.05% aqueous TFA, mobile phase B: 80:20% ACN:H_2O containing 0.04% TFA; gradient, 2–55% B; column temperature, 25 °C; UV detection at 214 nm.

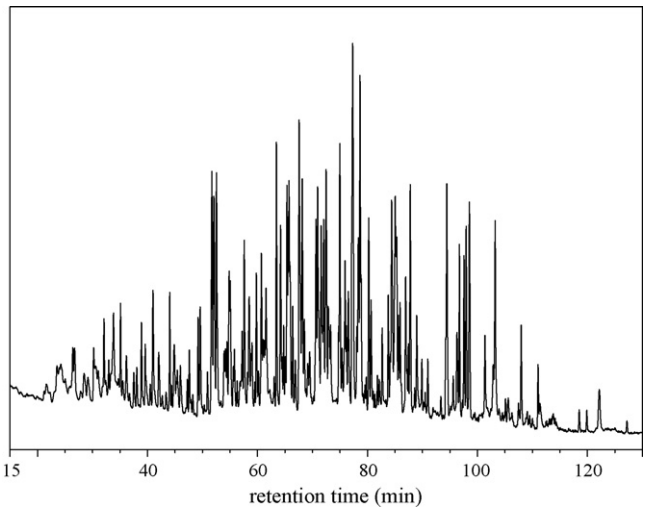

Fig. 3. Separation of a digest of a mixture of six proteins on a 50-cm column packed with 3-μm C18 particles at a gradient time of 120 min. Other LC conditions as in Fig. 2.

where t_G is the gradient time and W is the average peak width measured at 4σ (13.4% of the peak height for a Gaussian peak). Fig. 1 shows the effect of gradient time on the separation of a tryptic digest of a mixture of six proteins (transferrin, bovine serum albumin, β-galactosidase, alcohol dehydrogenase, lysozyme and cytochrome *c*), using a 150 mm 75-μm I.D. capillary column packed with 3-μm silica C18 particles at a column compartment temperature of 25 °C and applying gradient times of 15 and 60 min, respectively. The number of peptides resolved increased significantly when applying the slower gradient.

The peak capacity was experimentally estimated by averaging the peak width at 4σ for at least five peptides and applying Eq. (1). Fig. 2 shows the experimental effect of gradient time on peak capacity measured at different flow rates at a column temperature of 60 °C. At this elevated column temperature, diffusion in the mobile and stationary phase was enhanced, leading to faster mass transfer and, consequently, narrower peaks than when working at room temperature. At an even higher column temperature of 80 °C only a marginal increase in peak capacity was observed. Apparently, at high temperatures the change in the diffusion coefficients is smaller than that obtained in the temperature range from 20 to 60 °C. Furthermore, the trapping efficiency during desalting at high temperatures is decreased, potentially leading to a loss of the most hydrophilic peptides. Therefore, further experiments were conducted at 60 °C.

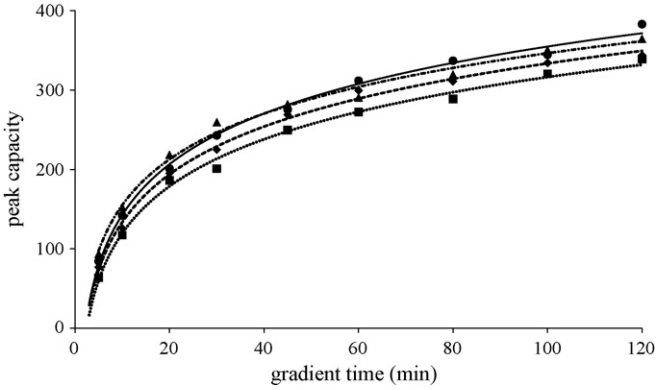

Fig. 2. Effect of gradient time on peak capacity using a 15-cm column packed with 3-μm C18 particles operating at a flow rate of 300 nL/min (■), 500 nL/min (♦), 750 nL/min (●), and 1000 nL/min (▲), respectively. Other LC conditions as in Fig. 1 except for column temperature, 60 °C. The lines are drawn to clarify the trends.

In general, longer gradient times yielded higher peak capacities. Under the present conditions (with $t_0 \approx 2$ min) a steep initial increase in peak capacity was observed when increasing the gradient time from 5 to 20 min. At longer gradient times the rate of increase was less steep and peak capacity tended to reach a maximum around 120 min, which is caused by an increase in the average peak width of peptides at longer gradient times.

When increasing the flow rate from 300 to 750 nL/min an increase in peak capacity was observed. Under these conditions the flow velocity is close to the optimum of the van Deemter curve, but much more importantly the gradient is a factor 2.5 "slower" in terms of column volumes (Fig. 2). At higher flow velocities the effect of the slower gradient diminishes and we enter the C-term region of the van Deemter curve, so that the peak capacity decreases at longer gradient times. These observations in the nano-LC mode match experimental data obtained on 2.1-mm I.D. columns [19].

To increase the peak capacity further, the effect of column length was also investigated. When using a 50-cm column and applying a gradient time of 120 min, a peak capacity of 464 was obtained (see Fig. 3). Under these conditions the t_0 increases 3.3-fold compared to that obtained on a 15-cm long column and a gradient of the same durations is effectively a much faster gradient than it is on the short column. When comparing the peak capacity per unit analysis time in one-dimensional gradient-elution LC of peptides for 15- and 50-cm long columns it is clear that it is profitable to apply longer columns. However, it should be noted that when using longer columns the total analysis time increases due to longer equilibration times.

3.2. Optimization of the off-line 2D-LC conditions

The first-dimension separation of peptides was performed on a polypeptide-coated strong cation-exchange column, applying a linear salt gradient at pH 3. The resin is negatively charged, while at this pH most of the tryptic peptides are positively charged. Since peptides with a lower p*I* bind less strongly to the cation-exchange resin than peptides with high p*I* value (isoelectric point), the peptides are depleted from the resin in order of increasing p*I* when increasing the salt concentration. In order to suppress hydrophobic interaction of the peptides with the polypeptide-

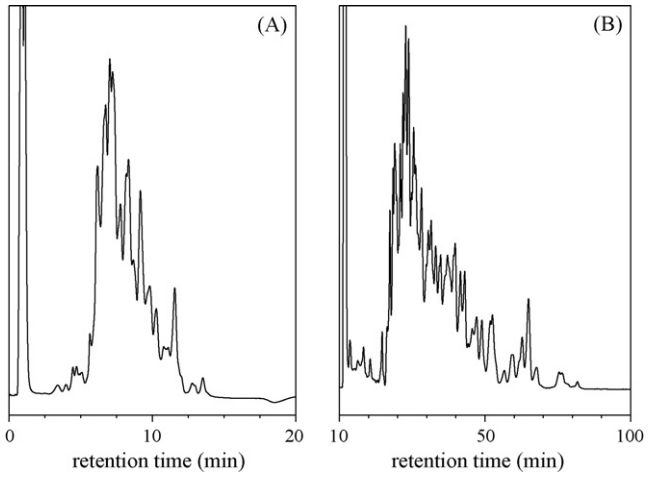

Fig. 4. Ion-exchange separation of a digest of a mixture of six proteins on a 15-cm column packed with 5-μm, 300 Å SCX silica particles, applying a linear gradient of 10 min (A) or 100 min (B). Other LC conditions: mobile phase A: 95:5% H_2O:ACN, 5 mM NaH_2PO_4 buffer pH = 3; mobile phase B: mobile phase A + 1 M NaCl; gradient, 0–55% B; column temperature, 25 °C; UV detection at 214 nm.

coated SCX phase, 5% of organic modifier was added to the mobile phase.

When applying a linear salt gradient the peak capacity in ion-exchange chromatography is defined as [26]:

$$n_c = 1 + \frac{\sqrt{N}}{4} \cdot (z+1) \ln\left(\frac{k_g}{z+1} + 1\right) \quad (2)$$

where N is the plate count, z the charge of the ion, and k_g the gradient retention factor (ratio of the retention time under gradient conditions (t'_R) and t_0). Eq. (2) shows that peak capacity is higher for peptides containing a higher charge and that peak capacity increases when peptides elute later (higher k_g value). In practice, the peak capacity can simply be adjusted by varying the gradient time (affecting k_g) or by optimizing column technology (affecting N), *i.e.* using longer columns or using a column of similar length packed with smaller particles. Fig. 4 shows the UV traces of the ^{1}D strong cation-exchange separation of a tryptic digest of a mixture of six proteins, applying linear salt gradients of 10 and 100 min, respectively. The resolution between the peptides increased when applying longer gradients, as was predicted by theory.

In order to use the full peak capacity in 2D-LC it was shown by Horie et al. that each ^{1}D peak must be sampled at 2.2–4 four times across its standard deviation (σ) [27]. The optimal sampling time for the first-dimension SCX separation of tryptic peptides obtained from a digest of cytochrome *c* was determined for a 10 and 100 min salt gradient (see Table 1). In practice, the lowest allowable fraction volume was around 6 μL, allowing a minimal sampling time of 10 s. Consequently, for fast ^{1}D runs the sampling rate would be too low, while applying long ^{1}D runs would results in an undesirably high number of ^{2}D fractions that would need to be analyzed.

When the sampling time exceeds the peak width of first-dimension peaks we speak of "undersampling", in which case the

Table 1
Determination of the optimal sampling time for tryptic peptides (based on eight fractions per 4σ peak width) when applying a salt gradient at 50 μL/min.

	Gradient time (min)	
	10	100
4σ peak width (s)	20	78
Optimal sampling time (s)	2.5	10
Optimal sampling volume (μL)	2	9
Number of fractions collected	240	600

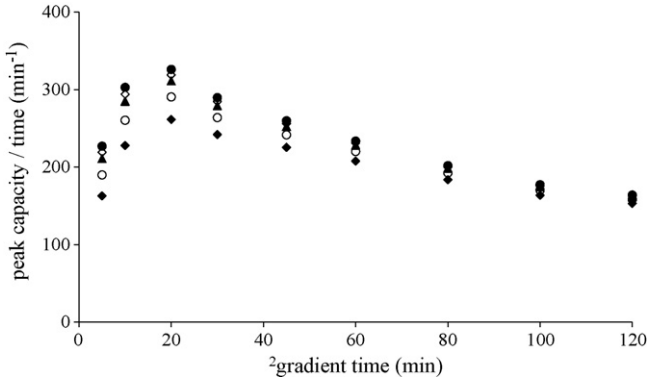

Fig. 5. Effect of ^{2}D gradient time on the 2D-LC peak capacity per unit time with a 15-cm long column packed with 3-μm particles. ^{1}D sampling time of 10 min (♦), 5 min (○), 2 min (▲), 1 min (◊), and 0.1 min (●). Other LC conditions: 1. ^1t_G = 10 min (IEX conditions as in Fig. 4), desalting for 5 min at 40 μL/min; 2. ^2t_G = 20 min, wash step at 90% B for 2.5 min, equilibration time = 12.5 min.

total peak capacity in the first dimension cannot be higher than the number of fractions taken (*i.e.* $^1t_G/s_t$), so that we find for the two-dimensional peak capacity (using the simplest for the second-dimension peak capacity):

$$^{2D-LC}n_c \approx \frac{^1t_G}{s_t} \cdot \frac{^2t_G}{W} \quad (3)$$

where ^1t_G is the gradient time of the first-dimension separation, s_t the sampling time, ^2t_G the gradient time of the second-dimension separation, and W the average second-dimension peak width at 4σ. The quickest way towards high ^{1}D peak capacities is to increase the number of fractions collected by reducing the sampling time. At "undersampling" conditions, increasing the peak capacity by changing the column length or particle size of the ^{1}D column is subsidiary to increasing the sampling time.

In Fig. 5 the peak capacity per unit time is plotted against the ^{2}D gradient time for different ^{1}D sampling times. The calculation was performed for a 10 min ^{1}D gradient and applying sampling times between 6 s and 10 min. The total analysis time was calculated by summation of the ^{1}D analysis time, and the ^{2}D analysis time, taking into account a preconcentration/desalting time (5 min, the ^{2}D gradient time, a wash step (2.5 min), and the column equilibration time (12.5 min)). It can be observed that the highest peak capacity per unit time (under the present conditions) is obtained when applying a 20-min ^{2}D gradient. The peak capacity per unit time decreases with increasing the ^{2}D gradient time above 20 min.

3.3. 1D- versus 2D-LC performance

Fig. 6 shows the trade-off in LC performance in terms of peak capacity and total analysis time for 1D-LC using 15 and 50 cm long RP columns and for an off-line SCX/×/RP separation approach employing a 15-cm ^{2}D column. The different coloured lines represent different ^{2}D gradient times using a 15-cm ^{2}D column packed with 3-μm C18 particles. As expected the peak capacity per unit time ratio is higher for 50-cm columns than for 15-cm columns (excluding column equilibration time for 1D-LC separations). It is evident from Fig. 6 that – under the present conditions – 1D-LC is superior to 2D-LC for separations requiring a peak capacity of 375 (t_G = 70 min) or less. This is illustrated by the typical one-dimensional separation of a digest of a mixture of six proteins on a 50-cm column (Fig. 3). Higher peak capacities can be generated in 1D-LC using longer gradients. However, the gain in peak capacity per time unit is less than what can be obtained using 2D-LC. For example, in order to generate a peak capacity of 450 1D-LC requires an analysis is 120 min, whereas the same separation performance

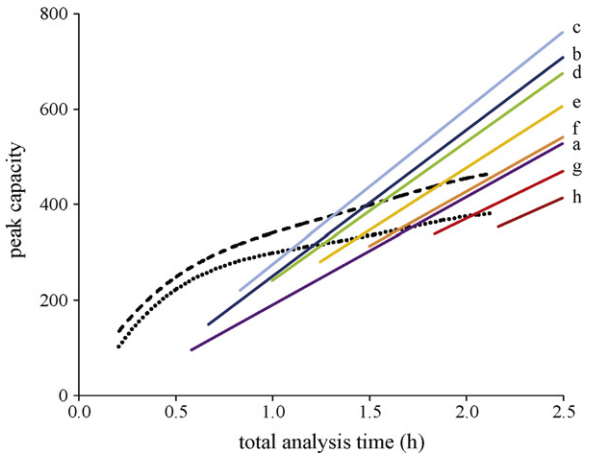

Fig. 6. Comparison of peak capacity and analysis time obtained in 1D and 2D-LC mode. 1D-LC data for a 15 cm (····) and a 50-cm nano-LC column (––) packed with 3 μm C18 particles. 2D-LC data applying a 10 min ^{1}D gradient and 5 min (a), 10 min (b), 20 min (c), 30 min (d), 45 min (e), 60 min (f), 80 min (g), and 100 min (h) ^{2}D gradient. Other LC conditions as in Fig. 5.

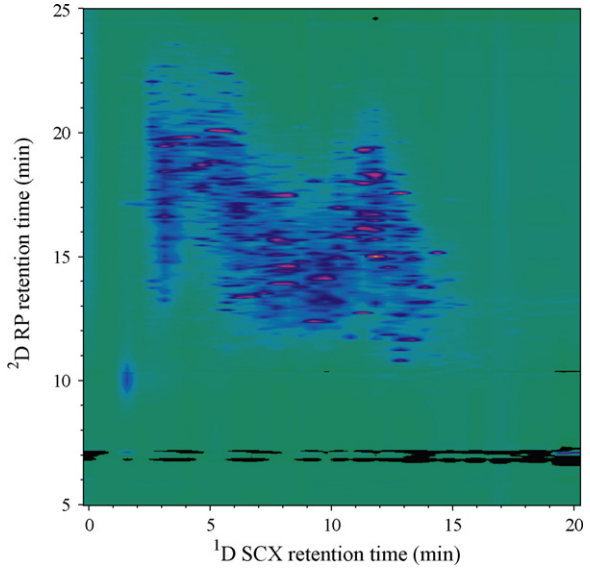

Fig. 8. Retention plot of the off-line SCX/×/RPLC separation of a tryptic digest of *E. coli*. ^{1}D SCX conditions as in Fig. 7 except for ^{1}injection volume = 2 μL, ^{1}D gradient = 4–55% B and collecting 0.5 min ^{1}D fractions during the 20 min ^{1}D analysis and ^{2}injection volumn = 2 μL.

can be obtained in 90 min using 2D-LC. The gain in peak capacity per unit time for 2D-LC becomes larger when higher peak capacities are demanded. It should be noted that the calculation assumes full orthogonality between the first- and second-dimension separations.

Fig. 7 shows the 2D-LC performance for the SCX/×/RP separation of a digest of a mixture of nine proteins, containing around 1000 peptides. The SCX fractions were collected and after precontration and desalting they were injected onto the ^{2}D RP nano-LC column and separated applying 20-min gradients. Fig. 7A shows the ^{1}D SCX separation. Peak-triggered fractions were collected from 8 min onwards. Fig. 7B and C show the corresponding contour plots when collecting 2 min and 1 min fractions, respectively. Co-elution of peptide fragments is evident in Fig. 7B. This is due to an inhomogeneous distribution of the peptides over the fractions. Fig. 7C shows a much better resolution due to the higher sampling frequency. However, it should be noted that that this gain in the theoretical peak capacity of 2000 was obtained at the expense of time (6 h total analysis time for Fig. 7C, *versus* 3 h for Fig. 7B). Furthermore, the sequence coverage obtained with MASCOT was only slightly higher with the 1 min sampling time compared to that obtained with a sampling time of 2 min.

The off-line SCX/×/RP separation of an *E. coli* digest is shown in Fig. 8. LC conditions were tuned such that a theoretical maximum peak capacity of 8720 could be obtained in a total analysis time of 26 h. In the ^{1}D SCX separation some of the single-charged hydrophilic peptides eluted under isocratic conditions before the start of the gradient. Consequently, the gradient and the sampling rate were adjusted, so that a uniform distribution of peptides was obtained over the 40 fractions collected for further analysis. Smearing of peptides over multiple fractions was not observed and an experimental 1D peak capacity of 38 was obtained in the 20-min SCX analysis. It can be observed that the tryptic fragments are randomly distributed within the elution window, illustrating the high degree of orthogonality of the SCX/×/RP approach.

Although this off-line 2D-LC method does not offer the required peak capacity to separate all peptides in an *E. coli* digest, around 500 000–600 000 peptides, within 24 h, the approach is useful to analyze complex samples. This is aided by the fact that it is not necessary to resolve all peptides for protein identification. The

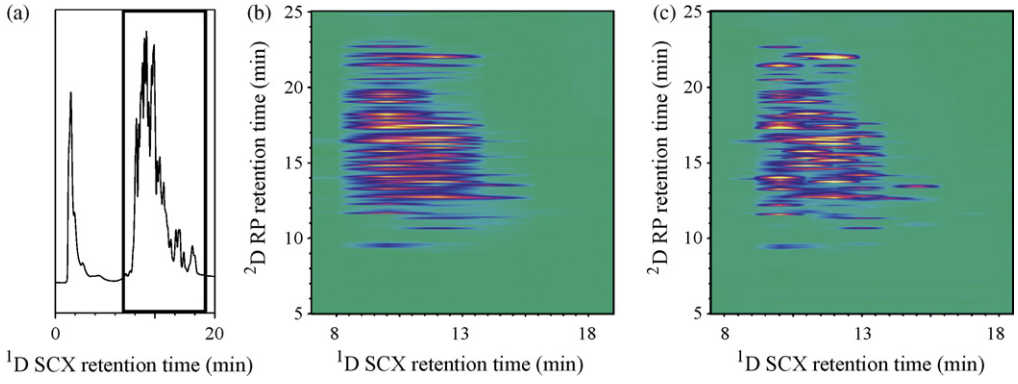

Fig. 7. 2D-LC separation of a tryptic digest of a nine-protein mixture obtained at ^{1}D sampling times of 2 (B) and 1 min (C). ^{1}D SCX separation depicted in (A). ^{1}D SCX conditions: injection volume = 10 μL, flow rate = 50 μL/min; mobile phase A: 95:5% H_2O:ACN, 5 mM NaH_2PO_4 buffer pH = 3; mobile phase B: mobile phase A + 1 M NaCl; gradient = 0–55% B; column temperature = 25 °C; UV detection at 214 nm. Fraction collection from 8 to 18 min. Desalting for 5 min at 40 μL/min; ^{2}D RP conditions: ^{2}injection volume = 20 μL, flow rate = 750 nL/min; mobile phase A: 0.05% aqueous TFA, mobile phase B: 80:20% ACN:H_2O containing 0.04% TFA; gradient = 2–55% B; gradient time = 20 min; wash step at 90% B for 2.5 min; equilibration time = 12.5 min; column temperature = 25 °C; UV detection at 214 nm, MS/MS detection via ESI interfacing.

resolution between peaks can be much smaller than unity when applying tandem mass-spectrometric detection.

4. Concluding remarks

Maximizing the peak capacity per unit time in one-dimensional LC involves using long gradient times at a flow velocity in the optimum of the van Deemter curve. Applying high column temperatures yielded a significant increase in peak capacity, while the column pressure was reduced allowing the use of longer columns. The greater resolving power of long reversed-phase nano-LC columns yielded high detection sensitivity, due to narrower peaks while the gradient volume stayed the same. The conditions for off-line 2D-LC were optimized so as to obtain the highest peak capacity in the shortest possibly analysis time. The quickest way towards a higher peak capacity in off-line 2D-LC at "undersampling" conditions is to increase the number of ^{1}D fractions collected. Increasing the peak capacity by changing the column length or the particle size of the ^{1}D column is subsidiary to increasing then sampling time.

Under the conditions of the present study, 1D-LC was superior to the off-line 2D-LC approach in terms of analysis time for separations requiring a maximum peak capacity of 375 (t_G = 70 min, L_{col} = 500 mm, d_p = 3 μm.). 1D-LC (in combination with tandem mass spectrometry) proved quite suitable for the separation of digests of mixtures of six proteins. For more demanding separations the 2D-LC approach is required. A tryptic digest of a mixture of nine proteins mixture was analyzed within 3 h. A tryptic digest of *E. coli*, containing approximately 4400 proteins, was separated using off-line SCX/ × /RPLC conditions, yielding a theoretical peak capacity of 10 000 within 30 h.

Nomenclature

^{1}D	first dimension
^{2}D	second dimension
k_g	gradient retention factor = t'_R/t_0
L_{col}	column length
n_c	peak capacity
N	plate number
RP	reversed phase
s_t	sampling time
SCX	strong cation-exchange
t_{dwell}	dwell time (gradient mixing volume)
t_G	gradient time
$t_{inital\ hold}$	holding time (isocratic conditions at start of gradient)
t_0	elution time of an unretained marker
t_α	time that first analyte peak is eluted after the gradient reaches the end of the column
t_ω	time that the last analyte peak is eluted before the end of the linear segment of the gradient reaches the end of the column
t'_R	retention time under gradient conditions
W	peak width at 4σ (13.4% of peak height)
z	ion charge

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Table A1
Approximate equations for peak capacity in reversed-phase gradient-elution liquid chromatography.

Conditions	Equation
$t_\alpha = t_{start}$ and $t_\omega = t_{end}$	$n_c \approx \frac{t_{end}-t_{start}}{W} + 1 = \frac{t_G}{W} + 1$
$t_\alpha \geq t_{start}$ and $t_\omega \leq t_{end}$	$n_c \approx \frac{\Delta t}{W} + 1 = \frac{t_\omega - t_\alpha}{W} + 1$
$t_0 < t_\alpha < t_{start}$ and $t_\omega \leq t_{end}$	$n_c \approx \frac{\sqrt{N}}{4} \ln\left(\frac{t_{start}}{t_\alpha}\right) + \frac{t_\omega - t_{start}}{W} + 1$
$t_\alpha \geq t_{start}$ and $t_\omega > t_{end}$	$n_c \approx \frac{t_{end}-t_\alpha}{W} + \frac{\sqrt{N}}{4} \ln\left(\frac{t_\omega}{t_{end}}\right) + 1$
$t_0 < t_\alpha < t_{start}$ and $t_\omega > t_{end}$	$n_c \approx \frac{\sqrt{N}}{4} \ln\left(\frac{t_{start}}{t_\alpha}\right) + \frac{t_{end}-t_{start}}{W} + \frac{\sqrt{N}}{4} \ln\left(\frac{t_\omega}{t_{end}}\right) + 1$

Appendix A. Peak capacity in gradient elution

The simplest equation for the peak capacity in gradient-elution LC is

$$n_c \approx \frac{t_G}{W} + 1 \quad \text{(A-1)}$$

This equation was applied for all peak capacity calculations in our study, and it is assumed that all peaks are approximately equally broad (the average 4σ band with W), that the first peak elutes (exactly) when the gradient reaches the end of the column (*i.e.* at a time $t_\omega = t_{initial\ hold} + t_{dwell} + t_0$) and that the last peak elutes (exactly) at the end of the linear gradient (*i.e.* at a time $t_\alpha = t_{initial\ hold} + t_{dwell} + t_0 + t_G$). The initial-hold time of the gradient and the dwell time of the instrument have the same effect on retention and band broadening in gradient-elution LC. However, $t_{initial\ hold}$ is usually programmed by the user in time units, whereas t_{dwell} is determined by the instrument and its configuration, as well as by the flow rate selected by the user. The column hold-up time (t_0) is also affected by the flow rate.

If the first analyte peak (denoted by α) is eluted after the gradient reaches the end of the column ($t_\alpha \geq t_{start}$) and the last analyte peak (denoted by ω) is eluted before the end of the linear segment of the gradient reaches the end of the column ($t_\omega \leq t_{end}$), than the effective window for the gradient is smaller than t_G, so that

$$n_c \approx \frac{\Delta t}{W} + 1 = \frac{t_\omega - t_\alpha}{W} + 1 \quad \text{(A-2)}$$

Especially in comprehensive two-dimensional liquid chromatography (LC × LC) it is essential that the gradients in both dimensions are cropped, so that t_α is not much larger than t_{start} and that t_ω is not much smaller than t_{end}. In LC × LC both conditions would fit in the category "comprehensive waste of time".

If the first analyte peak is eluted before the onset of the gradient (but after t_0; (*i.e.* $t_\alpha < t_{start}$) the and/or the last analyte peak is eluted after the end of the linear segment of the gradient (*i.e.* $t_\omega > t_{end}$), than the effective window for the gradient is larger than t_G and there is some additional peak capacity in the isocratic segments. Appropriate equations are summarized in Table A1. If peaks are eluted before t_0, then t_0 should be substituted for t_α in the equations given in Table A1 and the peak capacity of the exclusion segment may still be added, *i.e.*

$$n_{c,excl} \approx \frac{t_0 - t_{excl}}{W_{excl}} \quad \text{(A-3)}$$

where W_{excl} is the average width of the excluded peaks.

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