Increasing Peak Capacity for the Gradient Analysis of Protein Digests and other Complex Samples

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Complex samples, such as protein digests, may potentially contain hundreds of sample components. Characterisation of these challenging samples therefore requires the application of chromatographic techniques that can deliver exceptionally high resolving power. The structural diversity of sample components typically means that gradient analysis by reversed-phase is the method of choice. It is advantageous to optimise the peak capacity, to enable the separation of as many analyte peaks as possible within the specified gradient time. This article briefly examines how assessing key parameters, including gradient time and flow rate, can allow the chromatographer to optimise the peak capacity for the gradient separation of a complex sample. Additionally, an approach using multiple columns coupled in tandem is demonstrated. This is shown to provide exceptional analytical performance.

Introduction

Peak capacity (P_C) can be used to assess the separation efficiency provided by a gradient method. It is defined as the maximum theoretical number of components that can be separated, with a resolution of 1, within the specified gradient time. The peak capacity can be estimated by the following equation, where t_G is the gradient time and w is the average 4σ peak width (measured at 13.4% of the peak height) [1]:

$$P_C = \frac{t_G}{\bar{w}} + 1$$

For highly complex samples, it is desirable to maximise the peak capacity by optimising the analytical conditions, to achieve separation of the maximum number of sample components. For gradient analyses, the peak capacity is affected by the gradient time, along with parameters such as flow rate and temperature [1, 2]. Peak capacity can also be raised by increasing the efficiency of the column i.e. by increasing column length and/or decreasing the particle size. It can therefore be highly beneficial to assess the influence of these parameters when developing methods for samples that require very high separation efficiency. This article examines how investigation of gradient time and flow rate can be used to optimise peak capacity for peptide mapping experiments and demonstrates potential benefits that can be achieved through column coupling. The approaches discussed are also applicable to the analysis of other complex samples demanding high separation efficiency, such as natural products, complex biological mixtures and environmental samples.

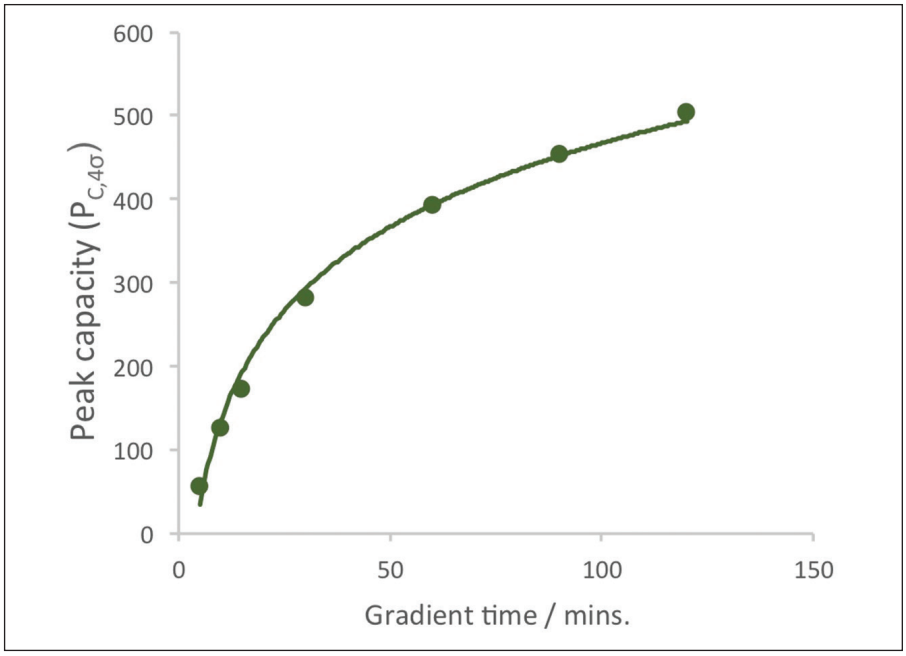

Figure 1: Effect of gradient time on measured peak capacity for the analysis of a BSA tryptic digest sample using a 10-40% B gradient at a flow rate of 0.21 mL/min on an ACE UltraCore 2.5 SuperC18, 150 x 2.1 mm. Mobile phase: A = 0.05% TFA in H_2O, B = 0.05% TFA in MeCN; Temperature: 60 °C; Detection: UV, 214 nm; Injection volume: 20 µL.

Assessment of gradient time and flow rate

Generally, increasing the gradient time will result in higher peak capacity, although at longer gradient times the increase becomes less significant as the peak capacity reaches a maximum [2]. To demonstrate this, a

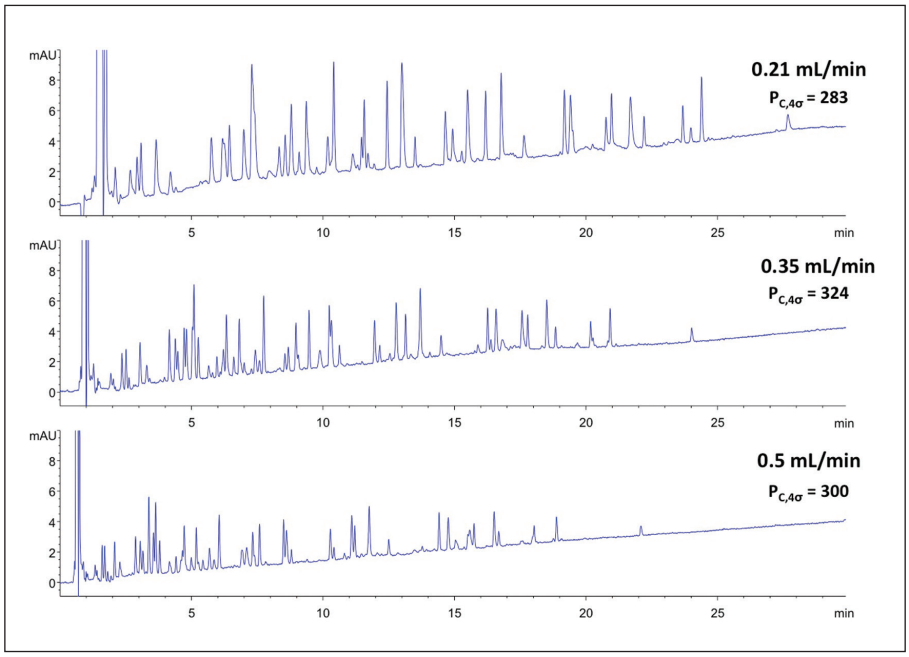

Figure 2: Comparison of peak capacity obtained for the BSA digest sample using a 30-minute gradient at three different flow rates on an ACE UltraCore 2.5 SuperC18, 150 x 2.1 mm. Mobile phase: A = 0.05 % TFA in H_2O, B = 0.05 % TFA in MeCN; Gradient 10-40% B in 30 minutes; Flow rate: 0.35 mL/min; Temperature: 60 °C; Detection: UV, 214 nm; Injection volume: 20 µL.

bovine serum albumin (BSA) protein digest sample was run on a 150 x 2.1 mm column packed with high efficiency 2.5 µm solid-core particles using a 10-40% B gradient. Various gradient times ranging between 5 and 120 minutes were assessed at a flow rate of 0.21 mL/min. Solid-core particles were selected, as they can provide high column efficiency at a modest backpressure; they are therefore ideally suited to the column coupling approach discussed later in this article. Peak capacities for each gradient time were calculated using the average peak width for 10 resolved peptides. Figure 1 demonstrates how the measured peak capacity increases with increasing gradient time, although the gains become less significant at longer gradient time. There will typically be a trade-off between obtaining optimum peak capacity and the use of an acceptable gradient time, based on the specific requirements of the application.

To examine the effect of flow rate, a 30-minute gradient time was selected as a good compromise between performance and speed (doubling the gradient time to 60 minutes would only result in a 39% increase in peak capacity). The gradient separation was then assessed at 0.21, 0.35 and 0.50 mL/min (Figure 2). The maximum peak capacity value (324) was observed at a flow rate of 0.35 mL/min for this separation. The optimum flow rate is often application dependent and may be influenced by the mobile phase composition, column temperature and analyte physicochemical properties. These results demonstrate that by evaluating flow rate and gradient time for a new gradient separation, significant gains in peak capacity can be obtained. It is worth mentioning that the selectivity of the separation may change when using this approach of varying flow rate and gradient time independently. Therefore, a higher peak capacity may not always provide better resolution of a specific peak pair [3].

Coupling multiple columns for further peak capacity gains

Increasing column efficiency by decreasing particle size or increasing the column length is an additional approach that can be used to further increase peak capacity. Due to the maximum operating pressures of 400-600 bar offered by conventional HPLC instrumentation, both these options have practical limitations. However, the higher-pressure capabilities of UHPLC instrumentation (up to 1,400 bar) opens up the possibility of coupling together multiple UHPLC columns, packed with highly efficient sub-2 µm or solid-core particles, to obtain ultra-high-performance separations. This results in higher peak capacity values with subsequent higher average resolution and reveals low-level analyte details in complex samples that may not otherwise be attained. Columns packed with solid-core particles offer a particularly attractive option for column coupling, as they generate comparable efficiencies to sub-2 µm particles at lower back pressure. Coupling >2 columns packed with sub-2 µm fully porous particles is also possible and can be facilitated by using low viscosity mobile phases containing acetonitrile and working at higher column temperatures [4].

To use this approach, the method requires translation so that the gradient time is scaled with the column length. This ensures that the selectivity of the separation is replicated using the longer column format. This can be easily achieved using freely available translation tools [5] or fundamental gradient theory [6]. To demonstrate this approach, the optimum conditions for the BSA digest separation in Figure 2 (30-minute gradient and flow rate of 0.35 mL/min) were translated from the single 150 x 2.1 mm column to three ACE UltraCore columns coupled together. The three 150 mm columns were coupled, using low-dead-volume UHPLC connectors and 0.13 mm ID stainless steel capillaries, to give a total column length of 450 mm. Translation of the gradient method to the new column dimensions gives a gradient time of 90 minutes. The resulting separation is shown in Figure 3. By coupling three columns in series, the peak capacity was almost doubled from 324 to 639, with a maximum pressure of 764 bar; well within the limits of modern UHPLC instruments. The impact of this increase in performance can be readily appreciated by examining the zoomed-in region of Figure 3. Enhanced resolution of analyte peak pairs is apparent, along with enhanced sensitivity. A notable increase in the level of baseline detail, with the resolution of additional sample components, demonstrates the value of the column coupling approach for the analysis of complex samples.

Conclusions

Reversed-phase gradient separations are a powerful tool for the analysis of highly complex samples such as protein digests. This short article has demonstrated how evaluation of gradient time and flow rate can help to optimise gradient separations and obtain higher peak capacities; to aid chromatographers in separating as many sample analytes as possible. The optimum analytical conditions are likely to be application dependent; therefore, it is advisable to assess these parameters during the development of new analytical methods.

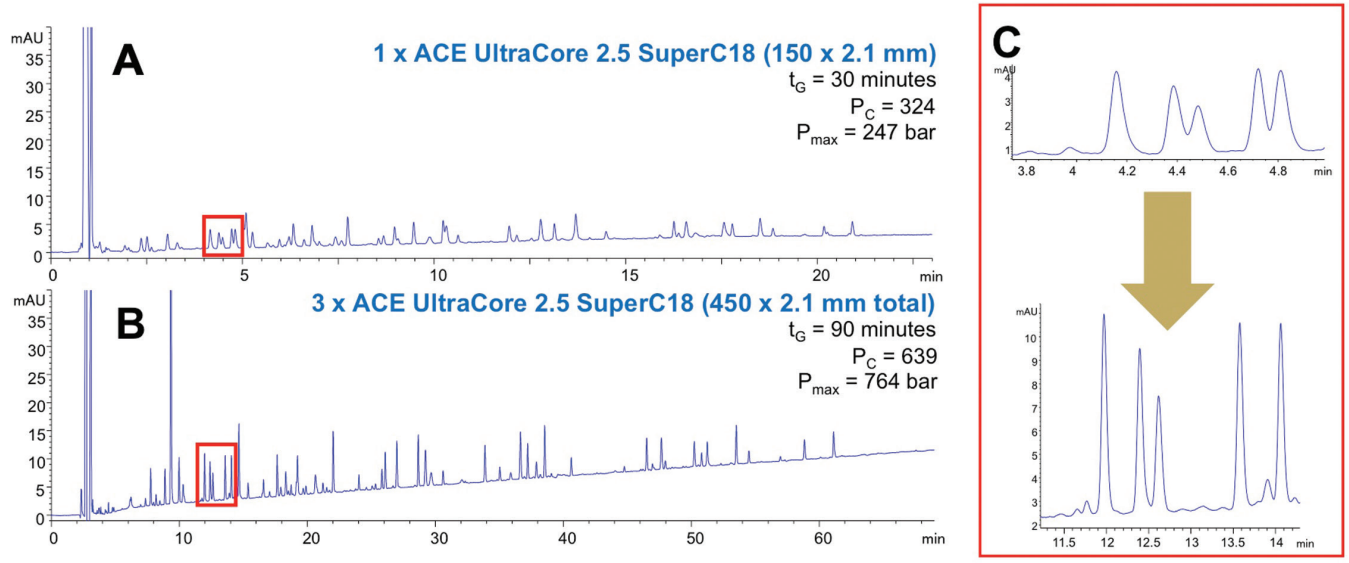

Figure 3: Gradient analysis of BSA protein digest sample on (A) one single 150 x 2.1 mm column packed with 2.5 µm solid-core particles and (B) three 150 x 2.1 mm columns connected in series. (C) Zoomed-in partial chromatogram comparing the regions highlighted in (A) and (B). Mobile phase: A = 0.05 % TFA in H_2O, B = 0.05% TFA in MeCN; Gradient 10-40% B; Flow rate: 0.35 mL/min; Temperature: 60 °C; Detection: UV, 214 nm; Injection volume: 20 µL.

The availability of UHPLC instrumentation means that coupling multiple columns, packed with high efficiency sub-2 µm or solid-core particles to obtain even higher peak capacities, is now a feasible option for many laboratories. Although this approach may require long analysis times, the potential advantages that are realised can make this an attractive option for the characterisation of highly complex samples.

References

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