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A practical guide to maximizing sample peak capacity for complex low molecular mass molecule separations.

복잡한 저분자량 분자 분리를 위한 시료 피크 용량 극대화 가이드

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Abstract

Method development for complex low molecular mass (LMM) samples using reversed-phase (RP) separation conditions presents significant challenges due to the presence of many unknown analytes over wide concentration ranges. This guide aims to optimize method parameters—column length (L), temperature (T), flow rate (F), and final mobile phase conditions (ø final)—to maximize separation peak capacity. Validated by prior research, this protocol benefits laboratories dealing with metabolomics, natural products, and contaminant screening.

This practical guide provides a structured approach to maximizing peak capacity for complex LMM separations. It complements computational optimization strategies and offers a step-by-step method development process. The Snyder-Dolan test is highlighted as essential for determining the need for gradient or isocratic elution and guiding column length decisions. The decision tree framework helps analysts prioritize variable optimization to develop effective separation methods for complex samples.

Keywords

LMM Separation, Snyder-Dolan test, Chromatography, Isocratic elution, Peak capacity

A practical guide to maximising sample peak capacity for complex low molecular mass molecule separations.

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INTRODUCTION

Method development for complex low molecular mass (LMM) samples using reversed-phase (RP) separation conditions is a challenging problem that typically requires gradient separation conditions, especially when the sample matrix itself may contain many unknown analytes present over a wide dynamic concentration range. This short article presents guidance based on an established approach published in 2013 aimed at optimising the practical method parameters (column length *(L)*, temperature *(T)*, flow rate *(F)*, and final mobile phase conditions ($\varnothing_{final}$) to maximise the separation's peak capacity[1,2]. The robustness of the protocol was verified in a previous study, and applied to optimise a highly complex maize seed extract sample[1]. This protocol may benefit the analysis of challenging samples with complex matrices in metabolomics, natural products and contaminant screening laboratories to name a few.

SAMPLE PEAK CAPACITY (n_c)

There are numerous peak capacity descriptors and variations in the way they are calculated[3,4]. Essentially, it is a metric that represents 'how many peaks from my sample can fit in my separation space/window?' Hence, the mathematical variations are associated with how the two (peak width and separation window) are measured and calculated. The sample peak capacity (n_c) approach where a large number of peaks is being separated, is based on chromatographic data via equation 1[3]

$$\text{Equation 1. } n_c = \frac{t_{R,last} - t_{R,first}}{4\sigma_{avg}}$$

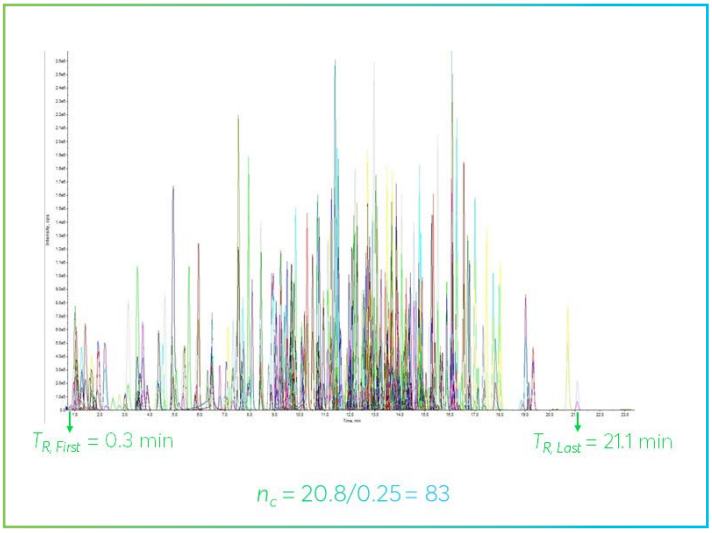

Figure 1: Calculating the sample peak capacity (n_c) based on a chromatogram (reproduced with permission of National Food Chain Safety Office, Directorate of Plant Protection, Soil Conservation and Agri-Environment, Hungary).[5]

Where the separation window is defined by the retention time difference between the last eluting ($t_{R,last}$) and the first eluting peaks ($t_{R,first}$). The maximum resolved number of peaks between them is defined by simply dividing the separation space or window by the average peak width measured within four standard deviations of the mean ($4\sigma_{aug}$); statistically $4\sigma_{aug}$ takes into account 99.9% of the population and therefore is a descriptor of peak shape and width.

Figure 1 shows how this is calculated from an actual chromatogram. The average peak width is 0.25 min, and the separation window/space was defined as 20.8 min, hence the sample peak capacity n_c equates to 83. Hence, a maximum of 83 baseline resolved peaks can fit within this defined separation space for this sample using this specific method.

The peak capacity can be used during method development to monitor method performance when changes are made, as well as to compare two methods to one another. Note that the sample peak capacity is representative of the LC separation strategy.

TRENDS IN n_c FOR LOW MOLECULAR MASS MOLECULES

The practical parameters and the complexity of the multivariate relationships associated with n_c has been previously studied with regards to relatively high molecular mass (HMM) analytes (peptides of a tryptic digest) and low molecular mass (LMM) species (representative set of indoles of a maize seed extract used for demonstrating the n_c for 2DLC studies)[6,7]. For further reading, please refer to the following references on this topic[1,2,8,9].

There are two main distinct differences between the HMM and LMM trends with regards to n_c[1,2,8,9]. One difference is related to the flow rate, which should be optimised for both HMM and LMM analytes. For HMM analytes, including peptides, the optimum peak capacity occurs at a lower flow rate that must be experimentally determined[8,9]. For LMM compounds, the increase in flow rate resulted in an increased n_c, and is related to the difference in the diffusion coefficients relative to larger peptides[1].

Another difference in trends between peptides and LMM species is that the column length is fixed and does not need optimisation for peptides. For LMM analytes on the other hand, column length must be optimised to maximise n_c. Hence, there is a difference in the practical strategy for maximising nc between peptides and LMW complex samples[1,8]. Furthermore, the success of the practical guide for maximising n_c for LMW complex samples, the Snyder-Dolan test is critical and is discussed in the next section of this communication[10].

The effect of temperature was also studied for three different search strategies utilising a free tool in Microsoft Excel 'Solver' to simulate method development experiments. Solver was instructed to maximise peak capacity, while simultaneously optimising (i) three practical variables (Φ_{final} , F, and L), (ii) two practical variables (Φ_{final} and F), and (iii) one practical variable only (Φ_{final}). All simulations had a fixed gradient time (t_G = 30 min). Temperature was also a fixed variable and set at 40, 60, 80, 100 and 120 °C for all three scenarios. Figure 2 highlights that an increase in temperature resulted in an increase in peak capacity and a maximum was reached near T = 80°C for optimisations (ii) and (iii). Figure 2 clearly shows that it is best to adopt search strategy (i) to optimise Φ_{final} , F, and L to achieve the best possible peak capacity. Hence, this strategy has been used to create the practical guide to maximise peak capacity for complex LMM samples. Not only does the column length and flow rate, as well as the final mobile phase composition (Φ_{final}), need to be optimised (so the last eluting species elutes at the end of the separation window), the temperature is also important and must be increased.

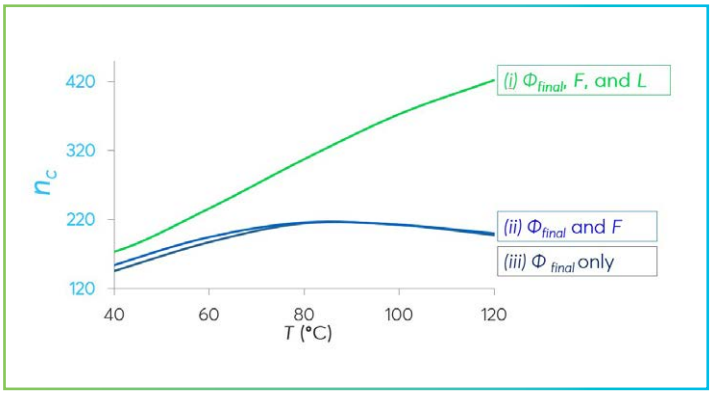

Figure 2: Effect of temperature for three simulated method development strategies where peak capacity was maximised: (i) three practical variables optimised (Φ_{final} F, and L), (ii) two practical variables optimised (Φ_{final} and F), and (iii) one practical variable optmised (Φ_{final})[1].

SNYDER-DOLAN TEST

The Snyder-Dolan (S-D) test is a critical aspect of this practical guide. An initial RP gradient screening run is performed that determines whether the complexity of the sample requires a gradient separation and additionally guides the column length choice[10]. The screen is conducted at 5-100% organic strength in 30 min, at 1 ml/min for a 4.6×100 mm column (dead time (t_0) of the column ≈ 1 min) at 30 °C. Adjustments can be made, for different column lengths or formats by multiplying t_G by the dead time of the column (i.e. 30×t_0). In addition, the flow rate

should be reduced for different column formats, as a basic guide approximately 1 column volume per minute (0.21 ml/min for a 2.1 mm ID column and 0.43 ml/min for a 3.0 mm ID column). To 'pass' the S-D test, the solutes must occupy more than 25-40% of the gradient time (t_G), where:

$$\text{Equation 3. } \frac{t_{R,last} - t_{R,first}}{t_G} \geq 0.25 - 0.40$$

Once the S-D passes the test to proceed with developing a gradient separation strategy, it is then recommended to follow the guide in the next section to maximise n_c. If the S-D test does not satisfy equation 2 (example in Figure 2b), isocratic conditions are recommended and the following protocol to maximise peak capacity may not be applicable.

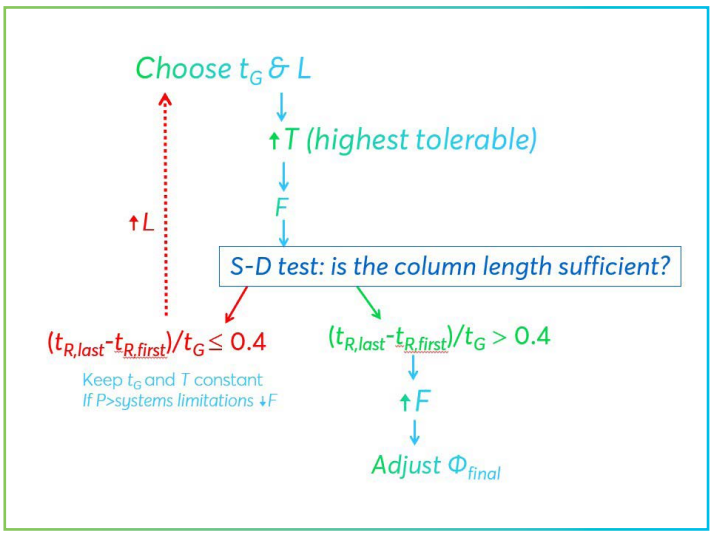

Figure 3: The decision tree aimed at maximising peak capacity for complex small molecule samples via RP gradient separation conditions [1].

A PRACTICAL GUIDE TO MAXIMISE n_c FOR COMPLEX LOW MOLECULAR MASS SEPARATIONS

Based on the previous study[1], the decision tree shown in Figure 3, is used to map out decisions and experiments in order of priority, based on the trends observed in the multivariate relationships between practical parameters when maximising peak capacity.

The first decision is intuitively related to the separation space, the longer the gradient time, the larger the peak capacity. **Note:** This is conducted after a column selectivity study and/or a final column selection decision is made. However, in the practical world, time is a constraint that is driven by the laboratory's productivity. Hence, while it may be an arbitrary decision, the gradient time is the first choice and must be guided by the priorities of the laboratory and how much time can be dedicated to analysing one sample. Hence, after setting t_G, the column length *(L)* must be selected. The most resourceful decision is to use what is readily accessible in the laboratory. Guidance on initial column lengths, based on different solute sets are provided in the previous study[1]. With regards to column selectivity and particle size selection - column selectivity and backpressure limitations must be considered and these must be fixed when conducting this protocol[11-13].

The next step is to select the highest temperature *(T)* possible that is compatible with the system, column and sample. If sample degradation is a concern during the analysis, a set of systematically different column temperatures can be tested to determine the highest temperature that is tolerable without compromising the integrity of the sample. If the temperature limits for the system and/or column are not clear - including the fittings and accessories - please refer to the care and use guidelines provided by the manufacturer.

The next step is to set the flow rate *(F)* at 1 ml/min (4.6 mm ID column) it can be scaled accordingly to other IDs and lengths (a free-method translator tool is available to **download** with a **how to use guide**). Next, the S-D experiment is repeated and if $(t_{R,last} - t_{R,first})/t_G \leq 0.4$ the column length must be increased while keeping the temperature and t_G constant, until $(t_{R,last} - t_{R,first})/t_G > 0.4$.

Once the correct column length is established, the flow rate is increased to the highest flow rate possible (compatible with the system and column), and the final mobile phase organic strength is adjusted so $t_{R,last}$ elutes $\leq t_G + t_0$. By following this guidance, all the available separation space is utilised, and both the separation space and peak width optimised for complex LMW samples separated via gradient RP conditions.

CONCLUSION

This short communication outlines how to maximise peak capacity for complex low molecular mass separations based on a previous study. This protocol is not presented to be used instead of computational optimisation strategies. It is aimed at providing a practical guide for analysts to follow to aid method development decisions for complex low molecular mass samples. We recommend using the presented decision tree that prioritises which variables to optimise first. This was developed based on the understanding of multivariate trends of practical parameters and peak capacity. Furthermore, the Snyder-Dolan test is critical for the success of this established protocol, to initially determine if the sample requires gradient or isocratic elution and to guide the column length decision.

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