Impact of column peak capacity on peptide mapping by reversed-phase high-performance liquid chromatography (RP-HPLC)

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Overview

- BioSuite™ columns for LC-MS and LC-UV peptide mapping
- Guidelines for column length (efficiency) selection (50, 100, 150 mm).
- Gradient selection guide (impact of gradient slope)
- Column peak capacity definition. Impact of peak capacity on peptide mapping.
- Role of selectivity in peptide mapping applications.
- General guidelines for fast peptide mapping method development

Introduction

Peptide mapping by RP-HPLC is often used for quality control of therapeutic proteins. Since tryptic digestion generates a complex mixture of peptides (typically 25-100, depending on the size of the protein), it is difficult to achieve an ideal separation of all peptides of interest. Separation optimization is often carried out intuitively by altering both column length and gradient slope. As a result, the method development for peptide mapping is a lengthy process.

A rational approach to RP-HPLC peptide mapping is suggested using a simple statistical model *(Davis and Giddings, Anal.Chem. 55, 1983, p. 418)*, predicting the number of peaks, *p*, resolved on column (observed in chromatogram) with resolution Rs=1.

$$p = m \cdot e^{-m/P_c} \qquad (1)$$

Assuming that tryptic peptides have random RP-HPLC retention (they are evenly distributed in the separation time (gradient time), *p* can be predicted from the sample complexity *m* (number of peptides in the separated sample) and the column peak capacity P_c. Peak capacity, as defined by equation 2, is the number of peaks that can be theoretically separated with resolution Rs=1 within a given gradient time.

$$P_c = 1 + t_g / w_{13.4\%} \qquad (2)$$

For example, when peak width at 4σ (13.4% of peak height) is 1 minute and gradient time t_g is 25 minutes, the peak capacity P_c=26. Peak capacity was measured experimentally (Figure 1) for BioSuite C_{18} 3µm PA-A and C_{18} 3.5µm PA-B columns using various gradient slopes.

Gradient slope *s* was calculated according to equation 3. Because the void volume V_0 changes with the column length, the gradient time has to be adjusted in order to keep the gradient slope constant for columns with different lengths. The Δc value is 1 for gradients from 0 to 100 % ACN. Gradients from 0 to 40 % ACN were typically used.

$$s = \Delta c \cdot t_0 / t_g \qquad (3)$$

Column Selection and Methods

BioSuite™ Peptide Analysis columns were selected for this study because of the their great peak capacity for peptide separation. BioSuite C_{18} PA-A columns are primarily recommended for LC-MS applications; they maintain high performance with MS compatible mobile phases comprised of aqueous formic acid (FA) and acetonitrile. BioSuite C_{18} PA-B columns perform best with trifluoroacetic acid (TFA) mobile phases (LC-UV applications). BioSuite PA-A columns are packed with silica based C_{18}, 3µm sorbent; in this study we used 0.1% aqueous FA as mobile phase A and acetonitrile with 0.065% FA (mobile phase B). BioSuite PA-B columns are packed with silica based 300Å C_{18}, 3.5µm sorbent; separation was performed with aqueous 0.1% TFA (mobile phase A) and 0.08% TFA in acetonitrile (mobile phase B). Flow rate was 1 ml/min, separation temperature 40 °C unless specified otherwise. Other conditions are shown in figures. The data were obtained using BioAlliance 2796 Separations Module and 2996 PDA detector. BioSuite columns, MassPREP™ peptide mix, Enolase digest, and Phosphorylase b digest are available from Waters.

Results and Discussion

Figure 1: Example of peak capacity measurement. BioSuite PA-B, 50, 100, and 150 x 4.6 mm, s=0.01. Peak capacity was calculated from equation 2. See also Table 1. According to the theory, that P_c increases proportionally to $\sqrt{N}$. Doubling the column length adds only 40 % to the peak capacity.

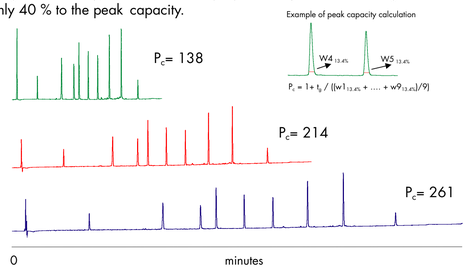


Figure 2: Impact of gradient slope on Enolase digest separation (50 peptides). The number of Enolase peptides resolved with R_s >1 is compared with column peak capacity measured as shown in Figure 1. Note that gradient slope also has an impact on the separation selectivity. Further extending the gradient time improves P_c and resolution only marginally. BioSuite PA-B, 50 x 4.6 mm column, for other HPLC conditions see Methods.

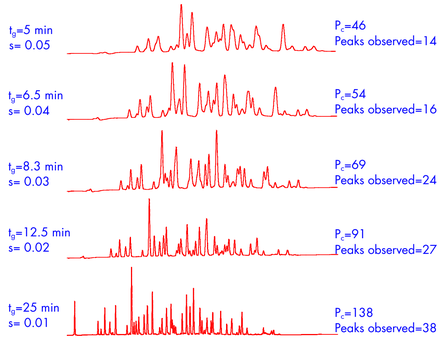


Figure 3: A correlation between number of predicted and observed peaks. Equation 1 is a useful tool for prediction of peptide mapping success providing that sample complexity *m* and column peak capacity P_c are known. The data for Enolase and Phosphorylase b are summarized it Table 1.

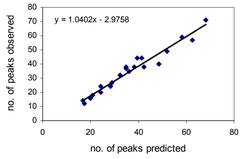


Table 1: Summary of P_c measurement/prediction data for Enolase (50 peptides) and Phsophorylase b (100 peptides).

Column type	L mm	slope s	grad. Time min	P_c measured 9 peptides	Predicted peaks equation 2 Enolase *	Peaks observed manual count, R_s>1 Enolase *	Predicted peaks equation 2 Phosphorylase**	Peaks observed manual count, R_s>1 Phosphorylase**
BioSuite PA-B	50	0.01	25	138	35	38	49	40
BioSuite PA-B	100	0.01	50	214	40	44	63	57
BioSuite PA-B	150	0.01	75	261	41	44	68	71
BioSuite PA-B	50	0.02	12.5	91	29	27	33	
BioSuite PA-B	50	0.03	8.33	69	24	24	23	
BioSuite PA-B	50	0.04	6.25	54	20	16	16	
BioSuite PA-B	50	0.05	5	46	17	14	11	
BioSuite PA-A	50	0.01	25	116	32	32	42	38
BioSuite PA-A	100	0.01	50	153	36	35	52	49
BioSuite PA-A	150	0.01	75	186	38	38	58	59
BioSuite PA-A	50	0.02	12.5	89	28	24	32	
BioSuite PA-A	50	0.03	8.33	70	24	20	24	
BioSuite PA-A	50	0.04	6.25	57	21	18	17	
BioSuite PA-A	50	0.05	5	47	17	12	12	

Figure 4: Adjusting the selectivity of peptide separation. (**A**) - by altering the separation temperature. (**B**) - by altering the type of C_{18} stationary phase. Both approaches result in substantial changes in selectivity and can be used for more convenient adjustment of peptide separation rather than changing the mobile phase composition or gradient slope. Columns: 100 x 4.6 mm (A) or 50 x 4.6 mm (B), s=0.01, for other conditions see Methods.

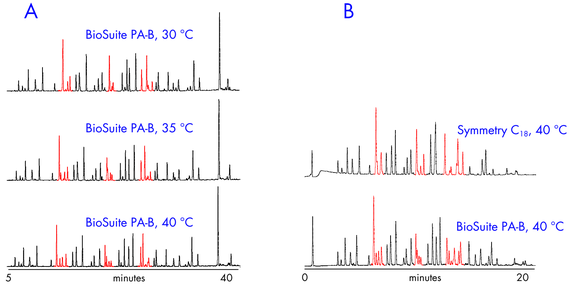


Figure 5: Separation of Phosphorylase b on BioSuite PA-B, 150 x 4.6 mm column, s= 0.01. About 71 peaks are resolved with R_s=1. From total number of Phosphorylase b peptides present, about 93 of them were at least partially resolved. A complete separation (if desirable) requires the use of a longer column and/or shallower gradient. Achieving a complete resolution by altering column selectivity or temperature (Figure 4) becomes extremely challenging for peptide maps consisting of >100 peptides.

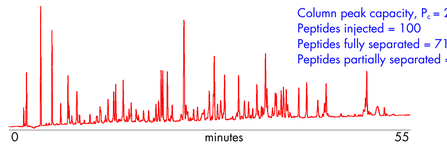
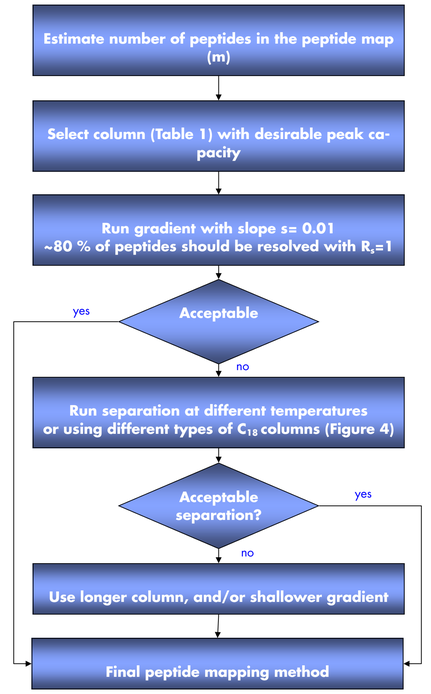


Figure 6: Guidelines for RP-HPLC peptide mapping method development.



Conclusions

- BioSuite PA-B columns with TFA mobile phases provide very high peak capacities.
- BioSuite PA-A columns maintain high peak capacity with MS compatible mobile phases (FA).
- Peak capacity is primarily important for the separation of complex peptide maps.
- Decreasing the gradient slope beyond s=0.01 improves peak capacity only marginally
- Changes in gradient slope change the separation selectivity. Transferring a method from column-to-column with constant *s* keeps the selectivity unchanged.
- A column with 4.4 x greater peak capacity than the number of peptides is likely to resolve 80% of peaks to the baseline. Remaining components will be partially resolved or unresolved.
- Use temperature or different type of RP-HPLC columns for fine tuning of separation selectivity.