Utilizing displacement effects in RP-HPLC purifications of peptides:

Applicability of a general method

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Introduction

The need for efficient peptide purification is ever rising due to growing importance of therapeutic peptides. Reversed phase HPLC has proven to be a powerful tool for peptide purification. Especially in early development, method optimization is not adequate due to restricted time and often lack of crude material to be spent on such experiments. Low initial purity of the peptide samples is typical fore early development and gives rise to very challenging separations.
In 2002, A. R. Mehok et. al.[1] presented a general RP-HPLC method that takes advantage of sample displacement chromatography (SDC). The proposed method applies two isocratic steps and renders high product purity and considerable productivity due to the high relative loadings that are necessary in order to obtain the required sample displacement effect.

Scope

The scope of this study was to elucidate if the proposed method delivers the results presented by Mehok et.al., for a variety of different peptides, or if there are restrictions to its general applicability. Also, different buffer systems were tested since these often are predetermined based on the further work-up. Within this work, three peptides of different sizes and initial purities were tested regarding application of the purification method proposed by Mehok et. al.

Summary of method principle[1]

- Determine the percentage X of organic modifier required to elute the product by applying a linear gradient
- Select 1st isocratic step at 20% less ACN than X → elution of hydrophilic impurities
- Select 2nd isocratic step at 15% less ACN than X → elution of product
  Regenerate at > 60% ACN → elution of hydrophobic impurities

Running conditions

- Load at 100% aq. for 30 min
- Run 1st isocratic step for 30 min
- Run 2nd isocratic step for 70 min
- Regenerate for 20 min

} Collect fractions

Adjustments to the original method

Within these studies, several adjustments to the original method were made. Different buffers were used since they were predetermined for each separation based on further work-up. The relative load was varied between different trials for each peptide separation. In this presentation, data for the best separations are presented.

Table1: Comparison of original and adjusted conditions

	Original	Adjusted
Buffer	0,05% TFA	various
Column	4.6x50 mm Discovery 180Å- 5µm-C18	2.1x150 mm Kromasil 100Å-5µm-C18
No of columns	3 in series	1
Linear flow rate during loading [cm/min]	3.0	2.9
Linear flow rate during elution [cm/min]	6.0	5.8
Load [mg/mL]	80	58-115

Experimentals

The columns were equilibrated at 100% buffer. In order to assure full wettability of the C18 column, a post column pressure of 26 bar was applied[2].

Table 2: Operating conditions for the peptide separations

	Peptide 1	Peptide 2	Peptide 3
Peptide size	10 aa	30 aa	18 aa
Buffer	0.1M NH_4Ac pH 4.5	0.05 M NH_4Ac pH 5.5	0.02M K_2HPO_4 pH 8.0
1st step	11% (v/v) ACN	31% (v/v) ACN	7.5% (v/v) ACN
2nd step	18% (v/v) ACN	36% (v/v) ACN	12.5% (v/v) ACN
Load [mg/g]	175	91	88

Results

The collected fractions were analyzed by means of a ACN gradient, and the elution profiles were reconstructed (Figure 1-3).

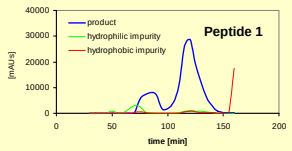

Figure 1: Reconstructed elution profile for separation of Peptide 1. Fraction analysis: KR100-5-C18 (4.6x250 mm), 0.16M KH_2PO_4 pH 5.15 / ACN 25/75 (v/v), 1.7 mL/min, 40°C

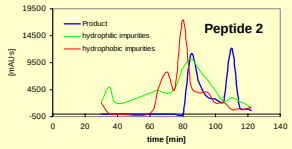

Figure 2: Reconstructed elution profile for separation of Peptide 2. Fraction analysis: KR100-5-C18 (4.6x250 mm), water / ACN + 0.1% TFA 31-46% ACN/ 35 min, 0.8 mL/min, 25°C

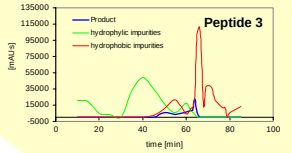

Figure 3: Reconstructed elution profile for separation of Peptide 3. Fraction analysis: KR100-5-C18 (4.6x250 mm), 20mM K_2HPO_4 pH 8.0 / ACN; 5-15% ACN/ 30 min, 1.0 mL/min, 40°C

Table 3: Purification results from SDC separation of the three peptides

	Peptide 1	Peptide 2	Peptide 3
size (aa)	10	30	18
Initial purity [%]	88	11	9
final purity [%]	94	42	28
recovery [%]	96	91	89
relative load [mg/g]	175	91	87

Discussion and Conclusion

Based on the presented results, it can be concluded that the method proposed by Mehok et. al. indeed renders good purification results for small peptides (peptide 1) at very high relative loadings without any preceding method development. Good purification results were obtained with buffers different to the TFA method described by Mehok et. al. For larger and significantly less pure peptides, the two-step isocratic elution scheme enabled an increase of the purity from ca 10 to ca 30 and 40% purity respectively (Table 3). These results are comparable to what can be achieved by shallow gradient elution methods that can be obtained from just a few experiments. It can be concluded, that highly non-pure crude peptides have to be purified by at least two orthogonal purification steps in order to obtain >90% purity even for the proposed isocratic two-step SDC method. Also, it can be presumed, that the isocratic two-step elution becomes less effective for peptides of increased size, due to the more pronounced dependency of % organic modifier on the retention time, slower diffusion, and slower adsorption / desorption kinetics.

As can be seen in Figure 1-3, the product elutes in two distinctive peaks. This behavior can be explained by the existence of multi-layer adsorption of the target peptide, rendering different retentions for the peptide-peptide and the C18 surface-peptide interaction. Multi-layer adsorption is commonly occurring if high sample loads are applied.

References

[1] A. R. Mehok et. al.; J. Chromatogr. A; 972 (2002) 87-99
[2] S. Winkel Pettersson et. al.; J. Chromatogr. B; 803 (2004) 159-165