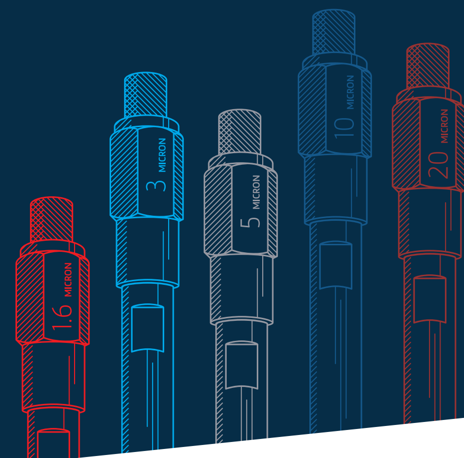
TECHNICAL PAPER

Orthogonality for Impurity Analysis of Synthetic Cyclic Peptides in Three Different Chromatographic Modes



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INTRODUCTION

Constrained peptides, such as macrocyclic and stapled peptides, have been drawing increasing attention as a promising class of compounds for the inhibition of protein-protein interactions (PPI).[1]

The analysis and purification of hydrophilic peptides is often performed by reversed-phase high-performance liquid chromatography (RP-HPLC). However, when the elution time of the target peptide and unwanted impurities are close or the same in RP-HPLC mode, it is difficult to accurately determine the purity of the target peptides.

Complementarity in chromatography is very effective for more accurate purity analysis (orthogonal analytical methods).[2]

This study aimed to examine the orthogonal relationship of synthetic cyclic peptide impurities in three different chromatographic modes; i.e. RP-HPLC mode, hydrophilic interaction chromatography (HILIC) mode, and supercritical fluid chromatography (SFC) mode. The crude cyclic peptides had neutral, acidic, and zwitterionic nature and were kindly provided by PeptiStar Inc. Three columns with synthetic polymer-based selectors, DCpak® P4VP,[3,4] DCpak® PTZ,[5] and DCpak® PMPC, were used for evaluation in HILIC and SFC mode (Figure 1). Particularly, DCpak® P4VP yielded a multitude of purification deliveries for challenging mixtures of highly polar drug substances, such as cyclic peptides.[4] Their chromatographic results were compared to RP-HPLC mode by using octadecylsilyl (ODS) stationary phase, and the orthogonality examined in detail.

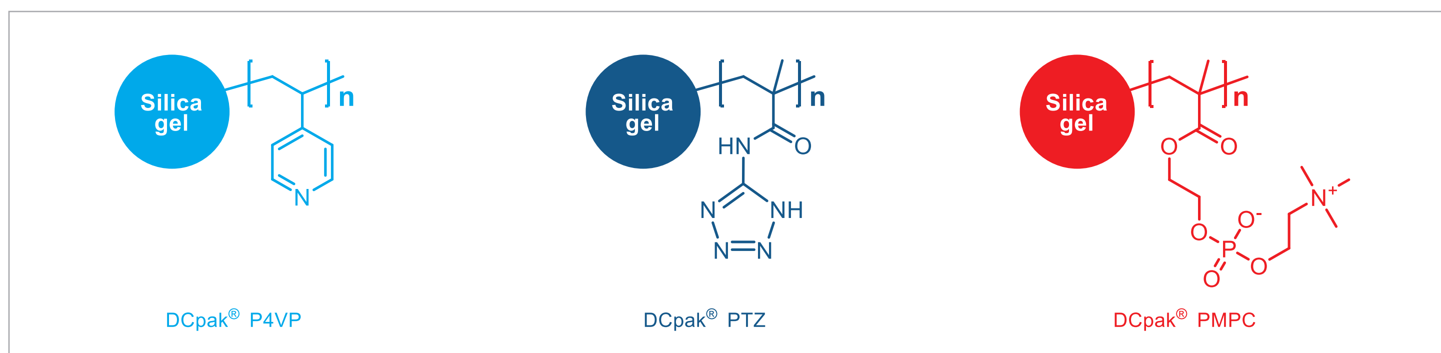

Figure 1. The structures of DCpak selectors used in this study.

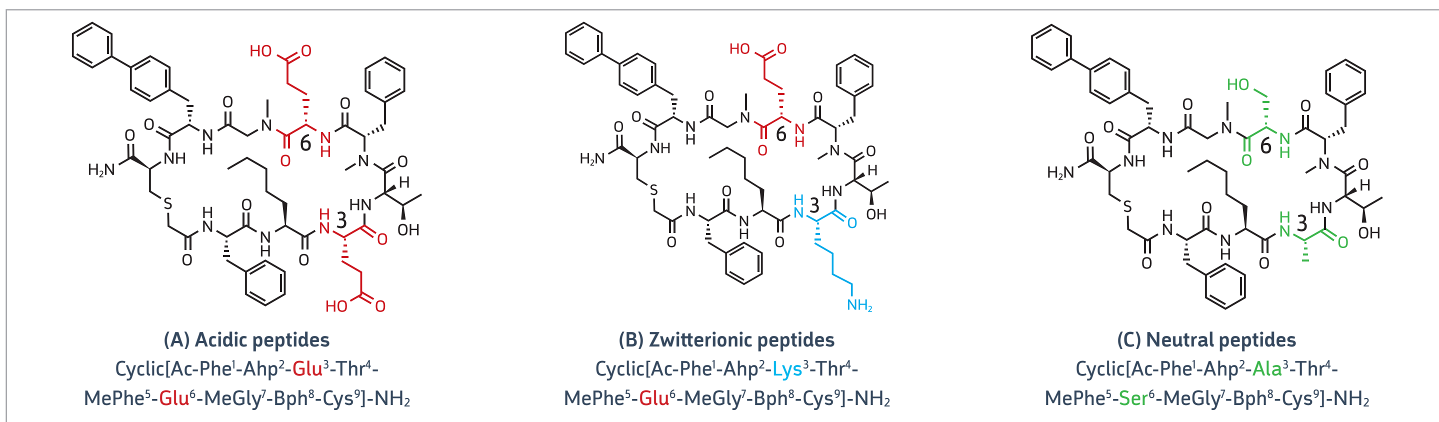

Figure 2. The structures of cyclic peptides used in this study. Peptides were crude and used as-is.

EXPERIMENTAL

The crude cyclic peptides used in this study were kindly provided from PeptiStar Inc (Figure 2). The solvents and reagents used in this study were all purchased from Kanto chemical Co., Inc. and FUJIFILM Wako Pure Chemical Corporation, and were used as-is. The RPLC instrument used in this study is Nexera X2 supplied by Shimadzu Corporation (Kyoto, Japan) equipped with pump, a vacuum degasser, a column oven, a multiple wavelength UV detector. The SFC instrument used in this study was a Nexera-UC supplied by Shimadzu Corporation (Kyoto, Japan) equipped with a CO_2 pump, a modifier pump, a vacuum degasser, a column oven, a multiple wavelength UV detector, and automated back pressure regulator (ABPR). Lab Solutions software (V 5.89) was used for system control and data acquisition. Chromatographic conditions, such as modifier, column temperature, ABPR pressure, total flow rate, detection wavelength, sample concentration, and injection volume are described in each figure (Figures 4-6), respectively.

DISCUSSION

The crude cyclic peptides were first subjected to a screening procedure to determine the suitable conditions for purification. As shown in Figure 3, the combinations of three DCpak columns and various mobile phase conditions in HILIC and SFC modes were investigated. In HILIC mode, acetonitrile (ACN) was selected as the organic solvent, and ammonium acetate with varying pH, phosphate buffer, acetic acid, and trifluoroacetic acid were used as additives, as these are typically used in HILIC analysis. For SFC mode, methanol is typically the preferred co-solvent; it is also well established that small portions (up to 5%) of water can be introduced to an alcohol co-solvent to improve peak shape and help the elution of compounds with high polarities.[6] Recently, ammonium hydroxide in water-rich modifiers ($MeOH/H_2O$ = 95/5) showed remarkable progress in separation of hydrophilic analytes such as cyclic peptide in SFC mode.[4] Based on these approaches, screening experiments were performed and from them, selected the most suitable conditions, which were chosen based on aspects of purity, peak symmetry factor, and theoretical plate number, etc.

Figure 4 shows the chromatograms of the crude acidic peptide

(A) HILIC Mode

✓ Column		✓ Experiment condition
DCpak P4VP DCpak PTZ DCpak PMPC	x	CH_3CN / H_2O CH_3CN / 20 mM AA* (pH 4.7) CH_3CN / 20 mM AA* (pH 7.0) CH_3CN / 20 mM AA* (pH 9.2) CH_3CN / 10 mM Phosphate buffer (pH 7.0) CH_3CN / 0.5% AcOH aq. (pH 3.1) CH_3CN / 0.1% TFA** aq. (pH 2.0)

*AA: ammonium acetate,
** TFA: trifluoroacetic acid

(B) SFC Mode

✓ Column		✓ Experiment condition
DCpak P4VP DCpak PTZ DCpak PMPC	x	CO_2 / MeOH CO_2 / MeOH / H_2O CO_2 / MeOH / H_2O (0.2% NH_3 aq) CO_2 / MeOH / H_2O (0.1% DEA#) CO_2 / MeOH / H_2O (0.1% TFA) CO_2 / MeOH / H_2O (0.1% Acetic acid) CO_2 / MeOH / H_2O (0.1% DEA# + TFA) CO_2 / MeOH / H_2O (20 mM AA)

#DEA: diethylamine

Figure 3. The combination of screening conditions (stationary phases and mobile phase conditions).

investigated in three different chromatographic modes, RP-HPLC, HILIC, and SFC modes. The target molecule eluted in ca. 12.9 min, 14.8 min, and 15.0 min in RP-HPLC, HILIC, and SFC modes respectively. The numbers represented in each chromatogram show impurities present in the crude samples. The same numbers represent the same impurities in all examples. Specifically, impurities 4 and 5 in RP-HPLC mode co-eluted with the target acidic peptide, but when the chromatography mode and/or column was changed, these impurities eluted farther from the target peptide. In HILIC mode when DCpak PMPC was used, these impurities eluted faster than the target peptide. In SFC mode, the same tendency was observed. It should be well noted that the same DCpak PMPC column used in HILIC and SFC mode produces different elution profiles, which can be of great significance depending on the application.

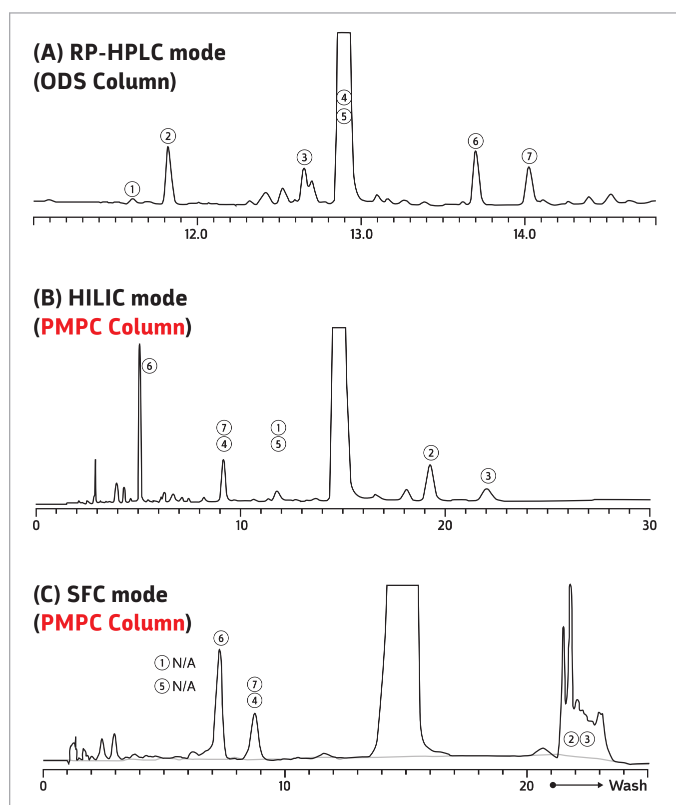

Figure 4. Chromatograms of crude acidic peptide in three different chromatographic modes. The peak numbers represented in each chromatogram show sample impurities. (A) RP-HPLC mode (ODS column) in gradient elution (TFA in water / TFA in CH_3CN). (B) HILIC mode (DCpak PMPC column, 3 µm, 4.6 x 250 mm) in isocratic elution (CH_3CN/H_2O/AcOH = 93/7/0.5, 1.0 ml/min, 40 °C). (C) SFC mode (DCpak PMPC column, 5 µm, 4.6 x 250 mm) in isocratic elution mode (CO_2/MeOH = 55/45, 3.0 ml/min, 40 °C, BP: 10 MPa).

For the zwitterionic peptide, DCpak PTZ was selected for HILIC mode and DCpak PMPC column for SFC mode. Similar to the acidic peptide chromatogram, impurities 3 and 4 eluted very close to the main peptide peak using the ODS column in RP-HPLC mode. However, with different chromatographic modes and different column chemistries, these impurities were found to be well resolved from the target peptide. Indeed, impurities 3 and 4 eluted later than the target peptide in HILIC and SFC mode (Figure 5).

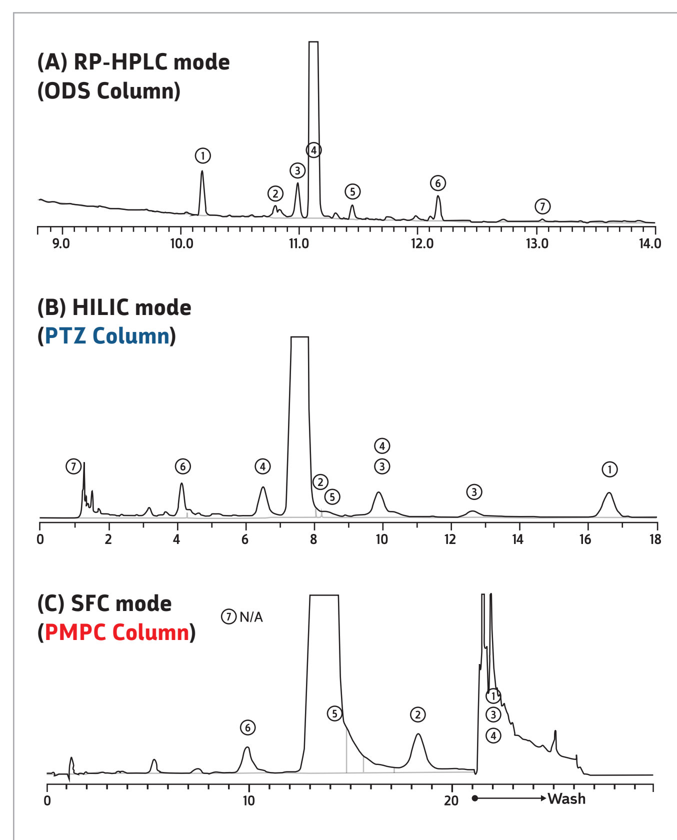

Figure 5. Chromatograms of crude zwitterionic peptide in three different chromatographic modes. The peak numbers represented in each chromatogram show sample impurities. (A) RP-HPLC mode (ODS column) in gradient elution (TFA in water / TFA in CH_3CN). (B) HILIC mode (DCpak PTZ column, 3 µm, 4.6 x 150 mm) in isocratic elution (CH_3CN/20 mM AA buffer (pH 4.7) = 85/15, 1.0 ml/min, 40°C). (C) SFC mode (DCpak PMPC column, 5 µm, 4.6 x 250 mm) in isocratic elution mode (CO_2/MeOH = 55/45, 3.0 ml/min, 40°C, BP: 10 MPa).

The opposite tendencies were also found to be possible. In the crude neutral peptide sample, impurities 2 and 10 eluted far from the target neutral peptide in RP-HPLC mode. When DCpak P4VP was used in HILIC and SFC mode, impurity 10 co-eluted with the target peptide. Impurity 2 was found

to eluted at different positions (before and after) from the target molecules, but relatively near to that in RP-HPLC mode (Figure 6).

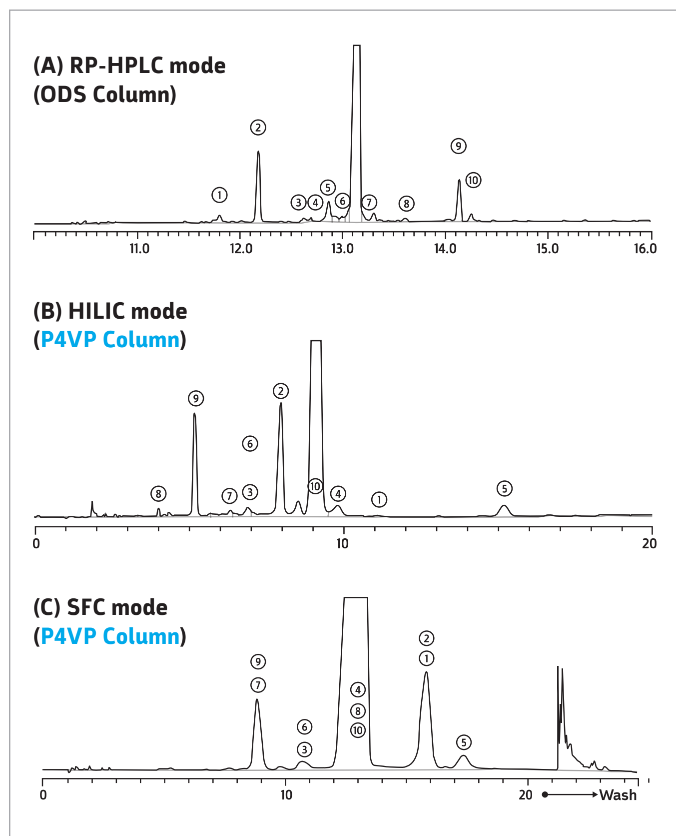

Figure 6. Chromatograms of crude neutral peptide in three different chromatographic modes. The peak numbers represented in each chromatogram show sample impurities. (A) RP-HPLC mode (ODS column) in gradient elution (TFA in water / TFA in CH_3CN). (B) HILIC mode (DCpak P4VP column, 3 µm, 4.6 x 150 mm) in isocratic elution (CH_3CN/H_2O = 97/3, 1.0 ml/min, 40°C). (C) SFC mode (DCpak P4VP column, 5 µm, 4.6 x 250 mm) in isocratic elution mode (CO_2/MeOH/H_2O = 75/24/1, 3.0 ml/min, 40°C, BP: 10 MPa).

CONCLUSIONS

In each peptide analysis, orthogonal trends compared to conventional RP-HPLC mode were observed. These results suggest that it is important to combine various stationary phases and/or chromatographic modes with classical RP-HPLC approaches (e.g., ODS columns). The use of complementary approaches allows for gained accuracy in analytical purity determination, but also improved preparative productivity and purity of the final peptide targets.

Acknowledgment

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