Evaluating Sustainable Ion-Pairing Agents in Peptide Chromatography

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Introduction

In peptide purification, mobile-phase acidity (available solution protons) strongly influences peptide charge-state distribution, retention, and separation selectivity. Trifluoroacetic acid (TFA) is widely used as an ion-pairing modifier due to its strong acidity and ion-pairing capability. However, environmental accumulation of stable trifluoroacetate salts have raised concerns for the long-term ecological impact of TFA in pharmaceutical workflows.[1] Growing emphasis to improve sustainability has driven interest in alternative modifiers such as formic acid (FA) and acetic acid (AcOH), which exhibit improved environmental profiles and reduced persistence.[2] Because these acids differ substantially in intrinsic acidity, their achievable proton activity may influence peptide protonation states and chromatographic performance. In the work presented here, Aib8-GLP-$1_{(7-36)}$-NH_2 (GLP-1) was selected as a mechanistically sensitive model peptide to evaluate how TFA, FA, and AcOH influence mobile-phase pH, proton activity, and chromatographic performance, Figure 1.[3]

***Figure 1**. Sequence of GLP-1 used in this evaluation. Ionizable sidechains are highlighted in blue or orange.*

Experimental Protocol

Synthesis

GLP-1 was synthesized on 0.4 mmol scale using a Biotage® Initiator+ Alstra™ using standard Fmoc conditions (5.0 eq. AA, 5.0 eq. Oxyma, and 5.0 eq. DIC; each at 0.5 M in DMF) at 85 ˚C for 2 min with the following adjustments: all His residues at 50 ˚C for 2 min; Leu residues at 85 ˚C for 10 min; and Arg residues at 50 ˚C for 10 min. Fmoc deprotection occurred using 4.5 mL of 40% 4-methylpiperidine in DMF 1 x for 30 sec and 2 x for 1 min. The resin was washed with 4.5 mL of DMF then 4.5 mL of DMF heated to 75 ˚C. The peptide was cleaved from the resin with 95% TFA: 2.5%TIPS: 2.5% H_2O for 3 hrs. The cleaved peptide was ether-precipitated and analyzed for purity using an Agilent 1260 Infinity series HPLC equipped with a Restek Raptor™ ARC-18 (2.1 × 50 mm) column connected to an Advion expression™ CMS mass spectrometer.

Purification

GLP-1 was dissolved in DMSO and purified using a Biotage® Selekt with a 10 g Biotage® Sfär Bio C18 column. The gradient consisted of 25 %B for 3 column volumes (CV), followed by 25-75 %B over 10 CV, and a final wash at 100 %B for 3 CV. Mobile phase consisted of MeCN/H_2O modified with 0.5% TFA, 0.5% FA, 0.5% AcOH, or 0.1% AcOH. Fraction collection was triggered using UV detection range of 200-220 nm.

Results and Discussion

Modifier Acidity Governs Proton Activity

***Table 1.** Modifier acidity comparison.*

Modifier	Measured pH value	Calculated [H^+]
0.5% TFA	1.6	2.6×10^{-2} M
0.5% FA	2.4	4.2×10^{-3} M
0.5% AcOH	2.9	1.4×10^{-3} M
0.1% AcOH	3.2	5.9×10^{-4} M

Measured pH values establish a clear hierarchy in acidity which may influence chromatographic behavior, Table 1. TFA produces a proton concentration that is 6-fold higher than 0.5% FA, 19-fold greater than 0.5% AcOH, and 44-fold greater than 0.1% AcOH. These results suggest that even at similar concentrations, alternative modifiers cannot achieve the same proton activity window as TFA. GLP-1 contains multiple ionizable residues, and reduced proton activity will likely alter charge-state uniformity and overall purification efficiency.

Effect on Chromatographic Peak Shape

To evaluate how differences in proton activity translate to chromatographic behavior, GLP-1 was purified as described and isolated fractions were analyzed by analytical HPLC, Figures 2 and 3.

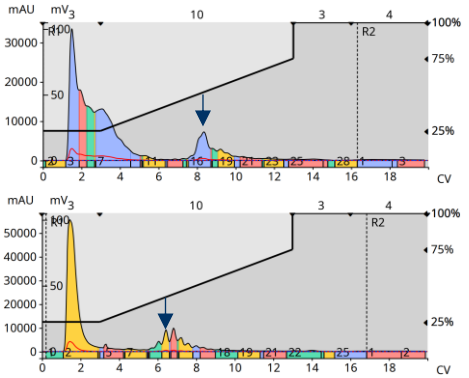

***Figure 2.** Representative RP-HPFC of GLP-1 with 0.5% TFA (top), 0.5% FA (bottom). Arrow indicates fraction analyzed for purity.*

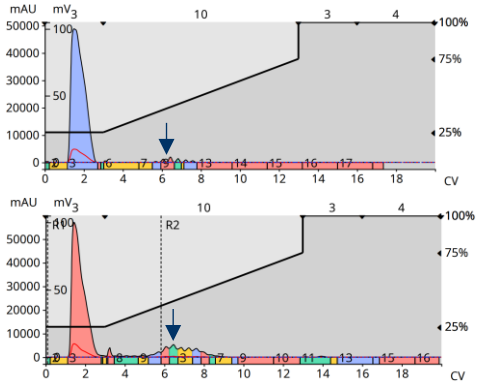

***Figure 3.** Representative RP-HPFC of GLP-1 with 0.5% AcOH (top), 0.1% AcOH (bottom). Arrow indicates fraction analyzed for purity.*

The hypothesis that lower proton activity would cause increased peak dispersion and broadened chromatographic profiles proved correct. Figure 4 displays an overlay of analytical HPLC traces of the purified product obtained under the different modifier conditions, illustrating the progressive peak broadening observed as proton activity decreases.

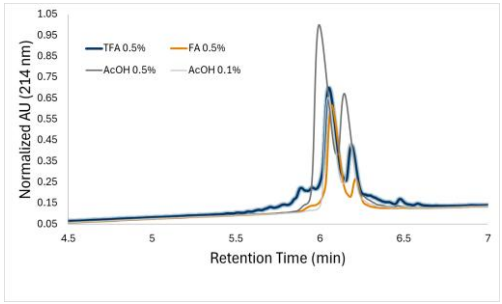

***Figure 4.** Overlay of analytical HPLC traces of the indicated fraction. isolated after GLP-1 purification using different modifiers.*

Recovery and Purity Trade-offs

The chromatographic differences observed during purification directly influenced isolated fraction quality. Fraction selection was guided by interpretation of the flash chromatograms (Figures 2-3) and verified by HPLC (Figure 4). Under 0.5% TFA, the product eluted as a single, dominant peak and the primary fraction was collected directly. In contrast, FA and AcOH systems produced increased dispersion, requiring analytical evaluation of multiple fractions to identify the fraction with the highest product purity and recovery.

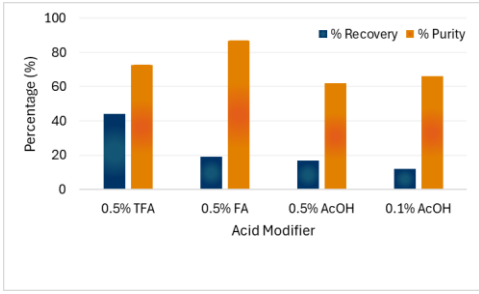

***Figure 5.** Comparison of recovery and purity across modifier conditions.*

To quantify these effects, peptide recovery and purity of the collected product fractions were evaluated across modifier systems, Figure 5. TFA provided the highest recovery, yielding 44% of the loaded peptide at 73% purity, while FA produces the highest purity of 87% with 19% of loaded peptide recovered. AcOH systems showed slightly reduced recovery of 17% and 12%, respectively, along with increased impurity co-elution due to lower proton activity conditions, reflected in reduced purity levels of 62% and 66%. These results demonstrate that modifier proton activity directly governs chromtographic performance. Stronger acids such as TFA maximize recovery through enhanced protonation and ion-paring, whereas intermediate acidity (FA) improves peak symmetry and product purity at the expense of recovery.

Conclusion

Acid modifier selection impacts charge-state uniformity and chromatographic selectivity during peptide purification. At 0.5%, TFA provided the most balanced single-fraction outcome, combining higher recovery with acceptable flash purity and a readily identifiable product peak, mirroring the crude analytical chromatogram. Formic acid produced the highest purity in the central fraction. However, adjacent fractions showed co-elution of product and impurities, limiting practical single-fraction recovery and simplified peak identification. Acetic acid conditions further increased peak heterogeneity and reduced separation between product and impurities. These findings suggest that while greener modifiers such as FA and AcOH reduce environmental burden, their lower achievable proton activity alters peak dispersion and impurity overlap. Sustainable modifier selection must therefore balance environmental impact with the level of proton control required to maintain both charge-state uniformity and chromatographic performance.

[1] Cabri, W., *et. al.*, Green Chem., **2022**, *24*, 975-1020.
[2] Redman, A., *et. al.*, Green Chem., **2015**, *17*, 945-949.
[3] Deacon, C.F., *et. al.*, Diabetol., **1998**, *41*, 271-278.

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