Greening Peptide Purification Through Use of Elevated Temperatures

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Abstract

With increasing global demand for peptide therapeutics, there have been significant efforts towards improving the sustainability of peptide production, to include both synthesis and purification. Even as much of the recent efforts towards greener and more efficient peptide purification workflows have focused on the development of continuous chromatography approaches (e.g. MCSGP), there remains a need to further improve batch chromatography approaches, particularly for lab scale purification. In this work, we explore the use of elevated temperatures (using a novel integrated heating system) to enable both improved purity and/or recovery as well as improved access to greener, though more viscous solvents, such as ethanol. A set of 11 crude synthetic peptides ranging from 9-42 amino acids in length (and a range of hydrophobicities and isoelectric points) were purified at elevated temperatures (60 °C) using either acetonitrile or ethanol as the organic modifier. Despite a minor loss of chromatographic efficiency using ethanol, the final purity and recovery were nearly identical when using either mobile phase (Acetonitrile: 95% purity, 55% recovery; Ethanol: 95% purity, 53% recovery), and significantly improved as compared to ambient conditions using acetonitrile (90% purity, 37% recovery). This finding in addition to similar eluotropic strength (average target elution of 26.1% acetonitrile, and 27.5% ethanol) and selectivity suggests wider applicability of the use of ethanol as a green solvent alternative, particularly in the context of elevated temperature conditions.

Integrated Heating System

Despite the established performance benefits of HPLC purification at elevated temperatures, including improved peak shape and solubility and lower overall system pressures, lab scale purification continues to be performed nearly exclusively at ambient temperatures. Traditional heating approaches (e.g. water baths and heating jackets) at the preparative scale are cumbersome and generally don't provide an effective means for eluent preheating, which is critical for maximizing peak shape and chromatographic efficiency (**Figure 1**) [1].

We present here a novel heating system (**Figure 2**; used in this work as a component of the Prodigy (CEM) preparative HPLC system) for semi-preparative and preparative applications, which is comprised of a forced air column oven with an integrated mobile phase heater. Rapid temperature equilibration along with easily accessible bulkhead fluid connections provide a facile means for accessing elevated temperatures, reminiscent of approaches commonly employed at the analytical scale.

Figure 1. Effect of Column Heating, With or Without Mobile Phase Heating, on Chromatographic Efficiency. A: Illustration Depicting the Influence of Temperature Mismatch (between mobile phase and column) on Band Broadening. B: Effect of Integrated Heating System on Resolution for 18-mer Crude Peptide

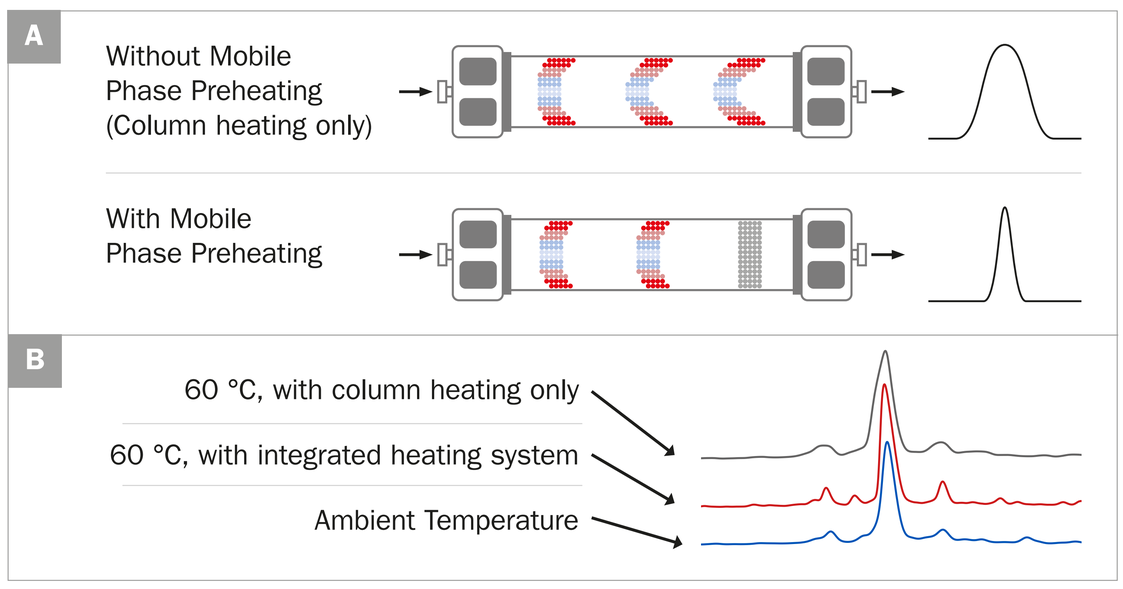
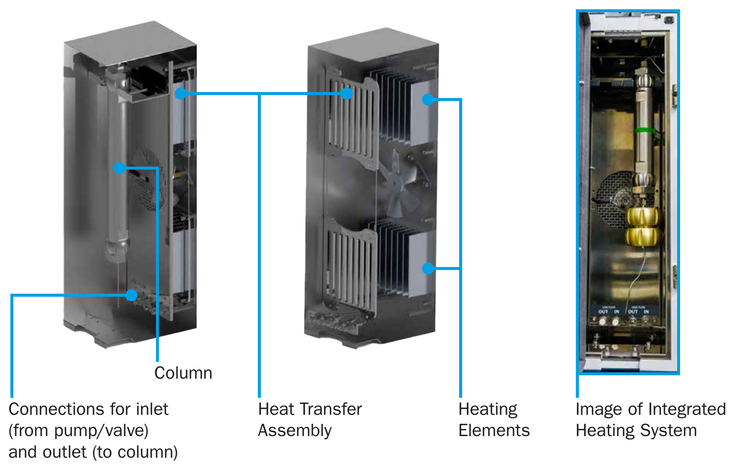


Figure 2. Renderings and Image of Novel Integrated Heating System



- Rapid temperature equilibration (< 15 min from ambient to 60 °C)
- Two alternative flow paths in heat transfer assembly (conductively heated by oven heating element), optimized for 10 mm or 19/21 mm ID columns
- Minimal contribution to system volume (< 0.2 mL for 10 mm ID flow path, and < 0.5 mL for 19/21 mm ID flow path)
- Operation up to 40 mL/min at 70 °C

Use of Ethanol for Purification at Elevated Temperatures

Ethanol has been identified as a greener alternative to acetonitrile for reversed-phase HPLC of peptides [2], due both to a similar eluotropic strength as well as a low UV cutoff (205 nm), but its significantly higher viscosity (1.1 cP, as compared to 0.38 cP for acetonitrile) has generally precluded application for routine lab scale purification. Particularly when using columns packed with 5 µm media (often used to achieve the required resolution, as compared to larger 10 µm media), the backpressure generated using ethanol in ambient conditions can exceed typical column pressure limits as well as place significant demands on HPLC hardware. System backpressure is directly related to solvent viscosity (**Eq. 1**), so the backpressure using ethanol can be nearly 3-fold higher than that using acetonitrile (**Figure 3**). Additionally, the increased viscosity results in slower diffusion ("*D*") in the mobile phase (**Eq. 2**), which can result in an increased resistance to mass transfer ("*C*") between the mobile phase and stationary phase. This can result in broader peaks and reduced resolution according to the Van Deemter equation (**Eq. 3**), whereby chromatographic plate height is directly related to the resistance to mass transfer. However, since viscosity is indirectly related to temperature, increases in temperature can reduce viscosity such that system backpressure can become more manageable (**Figure 3**) and the impact of broadening due to reduced mass transfer kinetics can be minimized.

In this work, a set of 11 peptides (9 – 42 amino acids in length) with a range of hydrophobicities and isoelectric points were screened on an analytical HPLC system using mobile phases with either acetonitrile or ethanol (denatured), both at 60 °C. The average elution % was nearly identical for both mobile phases (**Table 1**), enabling the use of the same (or similar) focused gradient methods for purification. Additionally, while the selectivity was similar for both mobile phases, there was a slight loss of efficiency (broader peaks) using ethanol due to the increased viscosity. However, after purifying each sequence on the Prodigy HPLC system (CEM) using 15 minute focused gradient methods (0.4%B/CV slope; custom gradients calculated according to analytical HPLC retention times), the final purities and recoveries using elevated temperatures were significantly improved over 25 °C conditions (90% purity, and 37% recovery), and the results were nearly identical when using acetonitrile (95% purity, 55% recovery) or ethanol (95% purity, 53% recovery) (**Figure 4**).

$$\Delta P = \frac{\eta F L}{K^0 \pi r^2 d_p^2} \quad \textbf{Eq. 1}$$

$$D = \frac{k_B T}{\eta} \quad \textbf{Eq. 2}$$

$$h = A + \frac{B}{v} + Cv \quad \textbf{Eq. 3}$$

Figure 3. Relative System Pressure Using Either Acetonitrile or Ethanol at Both 25 and 60 °C

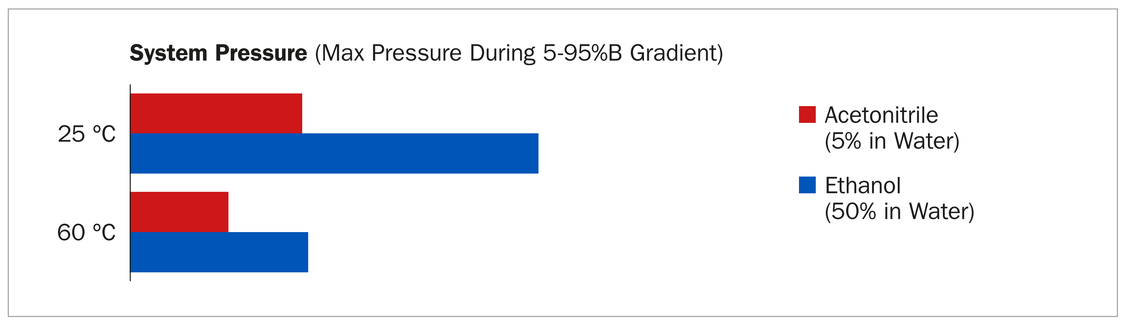


Table 1. Test Sequences and Analytical Elution %

#	Sequence	pI	Hydrophobicity	60 °C	
				Acetonitrile Elution %	Ethanol Elution %
1	GWVKPIIIGHHAYGDQYRAT	9.6	35.4	25.2	27
2	HGSRKNITDMVEGAKKANG	10.3	15.5	17.8	17.8
3	SLLNQPKAV	10.1	21.6	20.4	20.4
4	ALAVLSNYDA	3.1	25.2	24.7	25.8
5	TMEDKIYDQQVTKQCLCF	4.3	32.1	27.3	29.1
6	YSYPETPLYMQTASTSYYE	3.1	36.3	26.4	25.4
7	KVGYTERQRWDFLSEASIM	7.1	42.2	30.8	32.9
8	IVQENNTPGTYLLSVSARD	4.1	31.8	26.5	27.5
9	RFHMKVSVYLLAPLREALS	10.4	45.3	36.7	41.2
10	ENLKQNDISAEFTYQTKDA	4.1	29	21.1	21.9
11	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA ($^{1\text{-}42}$β-amyloid)	5.2	54.8	30.2 (70 °C)	34.0 (70 °C)
			Average Elution %	**26.1**	**27.5**

*#s 1-10 are neoantigen peptides described in a recent clinical trial for glioblastoma [3]

Figure 4. Screening and Purification at Elevated Temperatures Either Acetonitrile or Ethanol-Based Mobile Phases

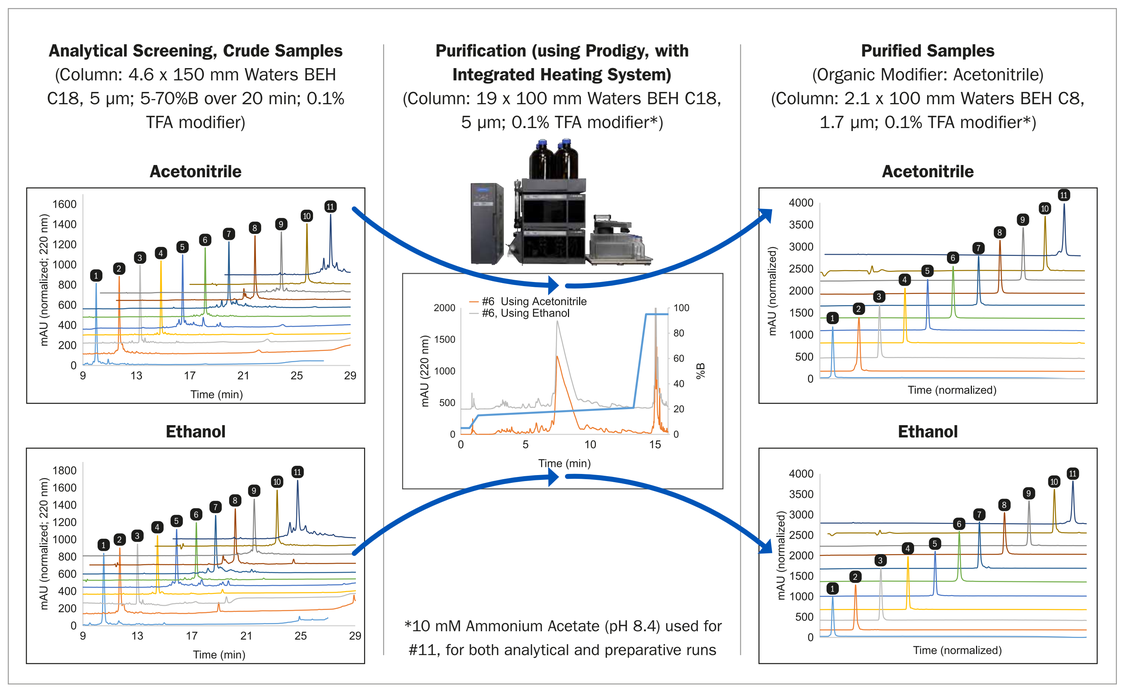


Table 2. Final Purity and Recovery

#*	Sequence	Crude Purity %	25 °C (150 mm column length; 1%B/CV gradient; 0.3% column loading)		60 °C (100 mm column length; 0.4%B/CV gradient; 0.5% column loading)*			
			Water / Acetonitrile Gradient		Water / Acetonitrile Gradient		Water / Ethanol Gradient	
			Purity %	Recovery %	Purity %	Recovery %	Purity %	Recovery %
1	GWVKPIIIGHHAYGDQYRAT	74	93	34	97	48	97	48
2	HGSRKNITDMVEGAKKANG	68	94	44	91	41	96	36
3	SLLNQPKAV	75	97	64	98	57	99	62
4	ALAVLSNYDA	79	95	66	97	63	95	72
5	TMEDKIYDQQVTKQCLCF	55	89	22	92	42	96	44
6	YSYPETPLYMQTASTSYYE	56	94	23	97	78	95	77
7	KVGYTERQRWDFLSEASIM	56	91	22	95	52	93	45
8	IVQENNTPGTYLLSVSARD	66	93	34	96	44	96	52
9	RFHMKVSVYLLAPLREALS	59	96	57	97	66	98	47
10	ENLKQNDISAEFTYQTKDA	71	87	30	93	52	91	46
11	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA	31	62	7	88	60	89	56
	Average	**63**	**90**	**37**	**95**	**55**	**95**	**53**

*Conditions for #11: 70 °C, 150 mm length column, 1%B/CV gradient

Conclusions

The application of elevated temperature conditions (using a novel integrated heating system) to enable the routine use of ethanol as an organic modifier for peptide purification was investigated. A set of 11 crude peptides (with varying lengths, hydrophobicities, and isoelectric points) were purified using either acetonitrile or ethanol-based mobile phases at 60 °C, and compared to purification using acetonitrile-based mobile phases at 25 °C. We found that purity and recovery were significantly improved using elevated temperatures and that the use of ethanol provided nearly identical results to that of acetonitrile, suggesting that any apparent loss of chromatographic efficiency using ethanol can be considered negligible in the context of purification using focused gradients and relatively high column loadings. Additionally, the eluotropic strength and chromatographic selectivity are nearly identical for ethanol and acetonitrile, suggesting wider applicability for the use of ethanol as an alternative green solvent.

References

[1] Brandt, A., Mann, G., and Arlt, W. Temperature gradients in preparative high-performance liquid chromatography columns. *J. Chrom A.* 769 (1997), 109-117.
[2] Brettschneider, F. et al. Replacement of acetonitrile by ethanol as solvent in reversed phase chromatography of biomolecules. *J. Chrom B.* 878 (2010), 763-768.
[3] Hilf, N. et al. Actively personalized vaccination trial for newly diagnosed glioblastoma. *Nature* 565 (2019), 240–245.