Superior Resolution of Peptides on SepTech ST150 10-C18 using Acetonitrile-free Gradient Elution

Application Note

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Introduction

Following recent developments in the chemical industry, and resulting global shortage in acetonitrile, Agilent Technologies has been investigating a range of alternative solvents that could be used for reversed phase HPLC analysis of peptides. One such solvent is ethanol, which has been shown in other application notes on this topic to give similar performance to acetonitrile.

Ethanol is a volatile, colorless liquid with a strong characteristic odor, that is miscible with water and with many organic solvents. As a relatively short, straight-chained alcohol, ethanol's hydroxyl group is able to participate in hydrogen bonding, rendering it more viscous and less volatile than less polar organic compounds of similar molecular weight (such as acetonitrile).

In this application note, a separation of 4 peptides is carried out using Septech ST150 10-C18 with both acetonitrile- and ethanol-based eluents, alongside comparative data from three leading competitor materials.

The SepTech ST150 10-C18 is a recently developed material based on high purity, totally porous, spherical silica that has a very narrow particle size distribution and, as you will be able to see from this application note, gives superior resolution of peptides under acetonitrile-free mobile phase conditions. It also offers savings in terms of the cost of materials when considering the scaling up of separations to preparative scale proportions.

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Materials and Reagents

Sample Preparation

A mixture of the following 4 peptides was made at a concentration of 1 mg/mL of each in a solution of 0.1% TFA in water:

Oxytocin	MW: 1007
Angiotensin II	MW: 2046
Angiotensin I	MW: 1296
Insulin	MW: 5808

Reference Chromatogram

Eluent A:	0.1% TFA in 20% ACN: 80% water
Eluent B:	0.1% TFA in 50% ACN: 50% water
Gradient:	0 – 100% B in 15 minutes
Flow Rate:	1 mL/min
Temperature:	Ambient
Injection Volume:	10 µL
Detection:	UV at 220 nm

With acetonitrile, there is very little background absorbance from the changing composition of the eluent throughout the gradient, therefore the baseline remains relatively stable for the duration of the run, as can be seen in Figure 1. All of the peptides elute in single sharp peaks, with very good resolution.

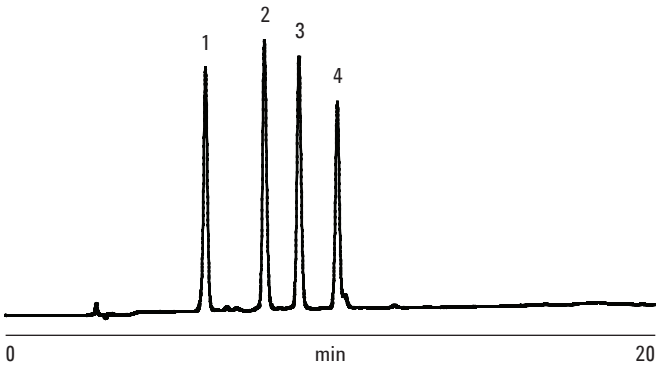

Figure 1. Peptide mixture on Septech ST150 10-C18 10 µm 250 x 4.6 mm ID column at 1.0 mL/min. Gradient elution of 0-100% B in 20 minutes. Compounds: 1. Oxytocin, 2. Angiotensin II, 3. Angiotensin I, 4. Insulin.

Results

Ethanol is a solvent that is sometimes overlooked for use in HPLC, however it has a low UV cut-off, and is very similar to acetonitrile in terms of solvent strength. Therefore, very similar gradient elution conditions were required to elute the peptides in a reasonable period of time, as shown below.

Eluent A:	0.1% TFA in 1% EtOH: 99% water
Eluent B:	0.1% TFA in 99% EtOH: 1% water
Gradient:	20 – 60% B in 20 minutes
Flow Rate:	1 mL/min
Temperature:	Ambient
Injection Volume:	10 µL
Detection:	UV at 220 nm

The use of ethanol results in a significant change in the selectivity of the Septech ST150 10-C18, as can be seen in Figure 2. Under gradient elution conditions, angiotensin I and insulin elute at very similar % B and are therefore partially co-eluted. The elution order has also changed, with insulin now eluting before angiotensin I.

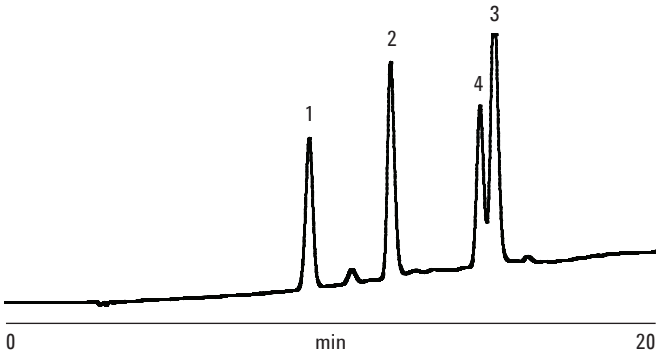

Figure 2. Peptide mixture on Septech ST150 10-C18 10 µm 250 x 4.6 mm ID column at 1.0 mL/min. Gradient elution of 20-60% B in 20 minutes. Compounds: 1. Oxytocin, 2. Angiotensin II, 3. Angiotensin I, 4. Insulin.

Ethanol also appears to have a greater UV absorbance at 220 nm, as the baseline drift during the gradient is much more significant.

Table 1. Comparison of column efficiency and peptide resolution for all solvents.

Solvent	Efficiency				Resolution (1,2)	Resolution (2,3)	Resolution (3,4)
	Oxytocin (1)	Angiotensin II (2)	Angiotensin I (3)	Insulin (4)			
SepTech ST150 10-C18	32,700	57,700	84,300	65,900	6.20	6.93	1.41
Competitor X	54,500	74,500	81,000	83,600	5.52	7.19	0.81
Competitor Y	26,400	38,700	27,800	71,000	2.91	6.73	0.45
Competitor Z	34,500	44,900	26,300		4.44	5.15	0.00

Competitor Column Comparison

For comparison, three competitor materials were also tested under the same gradient conditions with ethanol. The resulting chromatograms are shown in Figure 3.

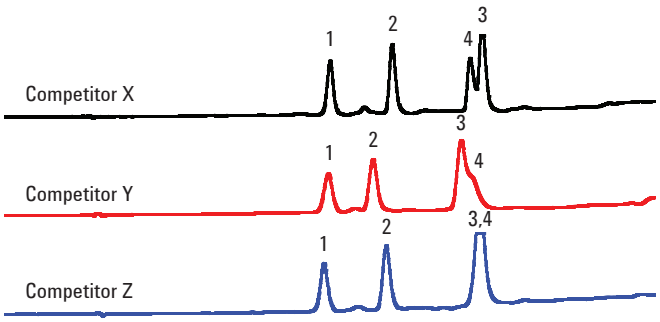

Figure 3. Peptide mixture on Competitor 10 µm 250 x 4.6 mm ID columns at 1.0 mL/min. Gradient elution of 20-60% B in 20 minutes. Compounds: 1. Oxytocin, 2. Angiotensin II, 3. Angiotensin I, 4. Insulin.

Table 1 summarizes the differences in the selectivity of the 4 different columns, in terms of the column efficiency and resolution between pairs of peptides.

Conclusion

These results show that ethanol can be used as an alternative solvent to acetonitrile for the reversed phase HPLC analysis of peptides. However, this does cause a change in column selectivity whereby insulin and angiotensin I start to elute at very similar % organic compositions and therefore partially co-elute.

Under a modified gradient elution profile, the Septech ST150 10-C18 gives better resolution between insulin and angiotensin than with three leading competitor columns. Competitor Z gave no separation at all between these two peptides.

All of the columns show some baseline drift due to the background absorbance of the ethanol when run with a UV detector at 220 nm. Therefore, an alternative detector such as a 385-LC ELSD could be considered which would easily evaporate this mobile phase and give flat stable baselines.

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