



# Investigation into the Alternatives to Acetonitrile for the Analysis of Peptides on a SepTech ST150 10-C18

## Application Note

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### Introduction

At present, laboratories worldwide are facing the prospect of having to re-develop many routine HPLC procedures due to a global acetonitrile shortage. A number of factors have contributed to this situation, and it is likely to continue for many months. According to a leading chemical supplier, acetonitrile is produced at relatively low volumes as a by-product from the manufacture of the monomer acrylonitrile, which is used to make its polymer, polyacrylonitrile; a fundamental raw material in the manufacture of plastics. Global demand for acrylonitrile is significantly reduced due to the slow down in consumer spending leading to production cut backs. Combined with global plant shut downs and outages, this has resulted in global acetonitrile raw material shortage.

This is causing a lot of problems as acetonitrile is the solvent of choice for most HPLC applications due to its low UV cut-off, low viscosity and good selectivity properties based on its relative polarity.

As a result, Agilent Technologies has been investigating a range of alternative solvents that could be used for reversed phase HPLC analysis of peptides, which maintain the efficiency and selectivity that SepTech ST150 10-C18 provides for this application when used with acetonitrile. This report discusses the findings of this investigation.



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## Materials and Methods

### Sample Preparation

A mixture of the following 4 peptides was made up to contain 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

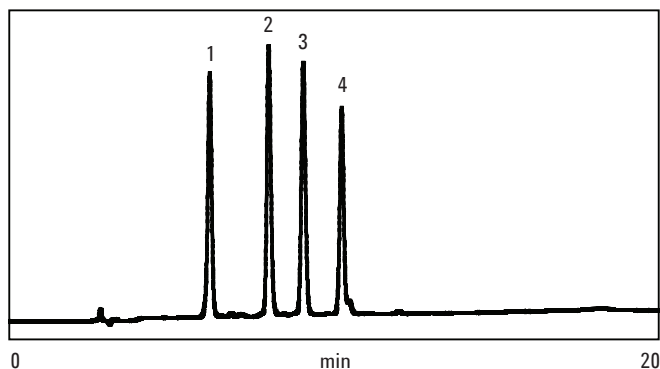
### Reference Chromatogram

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.

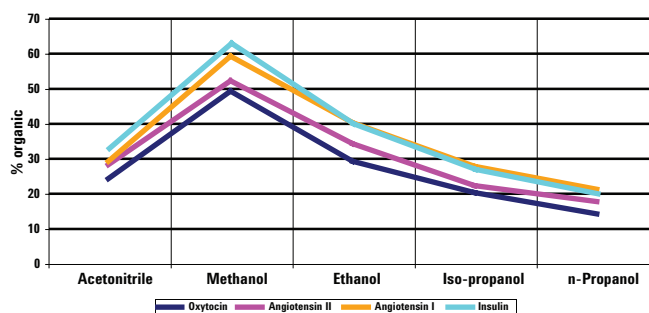


**Figure 1.** Peptide mixture on *Septech ST150 10-C18 10 µm 250 x 4.6mm id* column at 1.0 mL/min. Gradient elution of 0-100% B in 20 minutes. Compounds: 1. Oxytocin, 2. Angiotensin II, 3. Angiotensin I, 4. Insulin.

## Results

Four different solvents were replaced for acetonitrile, and the peptide mixture injected in each case. Due to the differences in the organic strength of each solvent, the start point and end point of the actual gradient profile needed to be modified to elute the peptides during the run.

This data was then used to calculate the percentage organic required to elute each peptide with each alternative solvent, which is summarized in the following chart (Figure 2):



**Figure 2.** Chart to show the % organic needed to elute each of the peptides with the *Septech ST150 10-C18*.

### Methanol

The first alternative solvent tested was gradient grade methanol, which is a replacement that is commonly made in the HPLC field. Methanol is less expensive than acetonitrile and has a low UV cut-off, but is a weaker solvent, therefore a higher proportion of methanol in the mobile phase is required for elution.

To obtain the data required for the plot in Figure 2, the gradient profile needed to be altered to the following:

Eluent A: 0.1% TFA in 1% MeOH: 99% water  
Eluent B: 0.1% TFA in 99% MeOH: 1% water

Gradient: 40 – 80% B in 15 minutes  
Flow Rate: 1 mL/min  
Temperature: Ambient

Methanol gives a similar separation to acetonitrile, however a greater proportion of organic is required in the mobile phase to elute the peptides. These elute between 49% and 63% methanol, as compared to 24-33% with acetonitrile.

This eluent has a high UV response at 220 nm, so the separation had to be run at a higher wavelength of 280 nm. With methanol, there is also the toxicity of the solvent to consider, particularly if methods are to be scaled up for preparative scale peptide purifications.

## Ethanol

Another, sometimes overlooked, alternative is ethanol, which also has a low UV cut-off but has twice the viscosity of methanol, and almost 4 times the viscosity of acetonitrile. Once again, mobile phase conditions needed modifying to account for the different strength of the solvent:

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

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. 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.

As with methanol, ethanol also has a higher UV absorbance at 220 nm and gives greater baseline drift and noise during the gradient.

## Iso-propanol

Iso-propanol (also known as propan-2-ol, IPA) is more commonly used as a modifier in normal phase chromatography, however it may also be used for reversed phase HPLC due to its miscibility with a large range of different solvents. Iso-propanol has 6 times the viscosity of acetonitrile and gave very high back pressure on our HPLC system. Increasing the temperature reduces the viscosity and operating pressure, and increases the efficiency of the separation.

The modified mobile phase conditions required to generate the data for the plot in Figure 2 are shown below:

Eluent A: 0.1% TFA in 1% iso-propanol: 99% water  
Eluent B: 0.1% TFA in 99% iso-propanol: 1% water

Gradient: 20 – 55% B in 15 minutes  
Flow Rate: 1.0 mL/min  
Temperature: 40 °C

IPA is a stronger solvent than ethanol or methanol, therefore less is required in the mobile phase to elute the peptides. However, this gives a similar separation to ethanol, with angiotensin I and insulin eluting at very similar % B. The elution order is also reversed compared to that of acetonitrile, and both are partially co-eluted.

Iso-propanol gives maximum UV absorbance at 204 nm, therefore under these conditions a high background

absorbance is obtained during the course of the gradient resulting in a noisy and drifting baseline.

## n-Propanol

n-Propanol (also known as propan-1-ol) is a primary alcohol like methanol and ethanol, and can also be used for reversed phase HPLC analysis.

As an isomer of iso-propanol, it has very similar physical properties but is slightly less viscous. It also needs to be run at 40 °C to keep back pressure down and improve overall column efficiency. The gradient profile was modified as follows to determine the elution profile for all four peptides:

Eluent A: 0.1% TFA in 1% n-propanol: 99% water  
Eluent B: 0.1% TFA in 99% n-propanol: 1% water

Gradient: 0 – 50% B in 20 minutes  
Flow Rate: 1.0 mL/min  
Temperature: 40 °C

n-Propanol is the strongest solvent of all four tested, therefore much less is required in the mobile phase to elute the peptides. However, once again, angiotensin I and insulin eluting at very similar % organic and are partially co-eluted (resolution between them is greater than with IPA and ethanol, however, as can be seen in Figure 2).

This solvent also gives baseline drift due to its absorbance at 220 nm.

Table 1 summarizes the differences in the selectivity of the 5 different mobile phase solvents, in terms of the column efficiency and resolution between pairs of peptides (under each modified gradient profile).

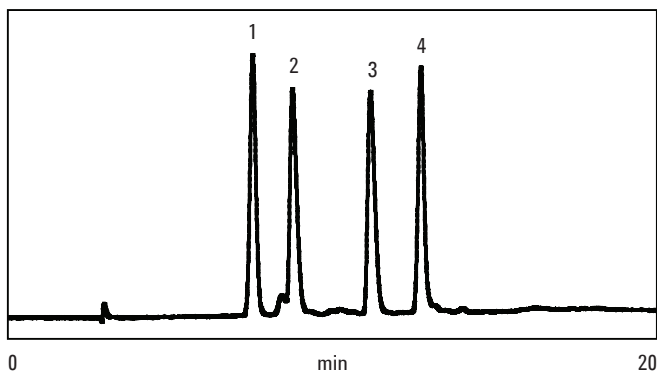
**Table 1. Comparison of column efficiency and peptide resolution for all solvents.**  
\* Elution order is (4,3).

Solvent	Efficiency				Resolution (1,2)	Resolution (2,3)	Resolution (3,4)
	Oxytocin (1)	Angiotensin II (2)	Angiotensin I (2)	Insulin (4)			
Acetonitrile	51,200	78,600	90,500	129,800	8.15	4.55	5.09
Methanol	32,400	33,000	48,700	93,600	3.40	6.09	4.23
Ethanol	32,700	57,700	66,000	84,300	6.20	7.30	0.88*
Iso-propanol	13,700	16,500	18,600	20,600	3.44	5.95	0.60*
n-Propanol	92,100	108,900	118,200	101,500	5.01	5.19	1.39*

## Conclusion

These results show that there are a number of different solvents that can be used as alternatives to acetonitrile for the reversed phase HPLC analysis of peptides.

With a Septech ST150 10-C18 column, the best alternative solvent for the peptides in our test sample is methanol which gave the greatest spacing between the peptides and a separation similar to that obtained with acetonitrile, as shown in Figure 3 below. However, the wavelength of the detector needed to be changed to 280 nm to avoid problems with high background absorbance. It must be noted that some peptides are more susceptible to degradation in acidic methanol.



**Figure 3. Peptide mixture on Septech ST150 10-C18 10  $\mu$ m 250 x 4.6 mm id column at 1.0 mL/min. Gradient elution of 40-80% B in 15 minutes. Compounds: 1. Oxytocin, 2. Angiotensin II, 3. Angiotensin I, 4. Insulin.**

Ethanol, iso-propanol and n-propanol all cause angiotensin I and insulin to elute at very similar retention times, therefore a separation between them is very difficult to obtain with these solvents. Iso-propanol and n-propanol also need to be run at elevated temperatures to reduce the viscosity and pressure, and improve the efficiency.

Most of these alternative solvents do introduce a certain degree of background absorbance 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 all of these different solvents and give flat stable baselines.

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