

Investigation into the Alternatives to Acetonitrile for the Analysis of Peptides

Application Note

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Introduction

At present, laboratories world-wide 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 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 a PLRP-S 100Å 10 µm provides for this application when used with acetonitrile. This report discusses the findings of this investigation.



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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.

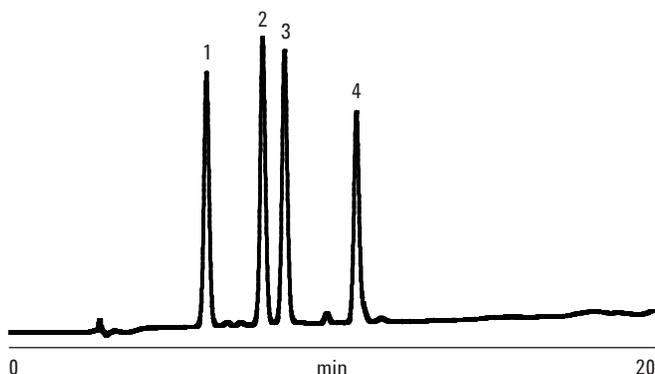


Figure 1. Peptide mixture on PLRP-S 100Å 10 µm 250 x 4.6 mm id column at 1.0 mL/min. Gradient elution of 0-100% B in 15 minutes. Compounds: 1. Oxytocin, 2. Angiotensin II, 3. Angiotensin I, 4. Insulin.

Results

Four solvents were evaluated as replacements 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, summarized in the following chart (Figure 2):

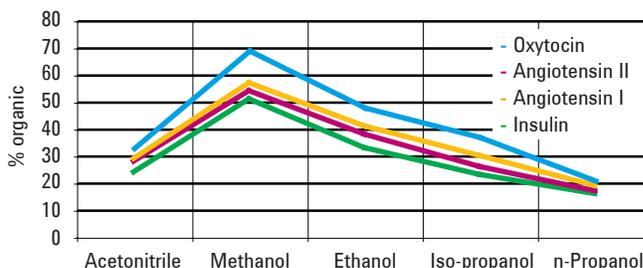


Figure 2. Chart to show the % organic required to elute each peptide with PLRP-S 100Å 10 µm.

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 very different selectivity on the PLRP-S column, with three peptides requiring between 51 and 57% B to elute, and insulin requiring 69%. This results in a separation whereby three peptides elute very close together, with insulin eluting much later.

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 eluent 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

With ethanol, the range in % organic required to elute the peptides is greater than with acetonitrile, which results in a slightly better separation. Overall, a greater proportion of organic solvent is required in the mobile phase to gain a separation.

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 water. Iso-propanol has 6 times the viscosity of acetonitrile and gave very high back pressure. 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, the spacing between them, in terms of the difference between the % organic required for the first and last peptide, is similar to that of ethanol.

IPA 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 less is required in the mobile phase to elute the peptides. However, as a result, all of the peptides elute at very similar % organic and are not as well separated. This solvent also gives baseline drift due to its absorbance at 220 nm.

Table 1 summarises 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.

Solvent	Efficiency				Resolution (1,2)	Resolution (2,3)	Resolution (3,4)
	Oxytocin (1)	Angiotensin II (2)	Angiotensin I (3)	Insulin (4)			
Acetonitrile	25,200	40,200	44,000	65,400	5.61	2.14	6.75
Methanol	20,600	25,300	31,300	54,200	1.87	1.69	8.70
Ethanol	27,800	37,200	40,500	53,000	4.53	2.67	5.36
Iso-propanol	10,200	10,100	15,300	9,800	3.45	2.95	4.04
n-Propanol	78,700	80,300	88,100	82,800	1.80	2.20	3.22

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 PLRP-S column, the best alternative solvent for the compounds in our test sample is iso-propanol which gave the greatest spacing between the peptides, as shown in Figure 3 below. However, due to the higher viscosity this solvent (and n-propanol) required elevated temperatures, or lower linear velocities/flow rates to avoid back pressure issues.

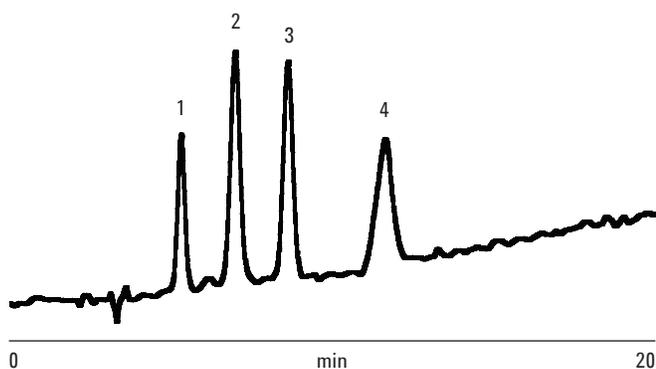


Figure 3. Peptide mixture on PLRP-S 100Å 10 µm 250 x 4.6 mm id column at 1.0 mL/min, 40 °C. Gradient elution of 20-55% B in 20 minutes. Compounds: 1. Oxytocin, 2. Angiotensin II, 3. Angiotensin I, 4. Insulin.

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