



Peptide analysis on Agilent PLRP-S with ELS Detection and Acetonitrile-free Eluents

Application Note

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Introduction

Due to the current global acetonitrile shortage, 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 gives for this application when used with acetonitrile. The details of this investigation can be found in Application Note 5990-7740EN.

One of the disadvantages of changing from acetonitrile to an alternative solvent is the increased background absorbance of the mobile phase eluents when run on a UV detector. Many solvents, such as methanol, ethanol, and iso-propanol, absorb UV strongly in this region giving baseline drift and noise that interferes with the separation, therefore this report details how a 385-LC ELS detector can be used to prevent this. The 385-LC ELSD is capable of evaporating a wide range of different solvents, resulting in completely flat baselines and sharp peaks, even during gradient elution.



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Materials and Reagents

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

Results

Two different solvents were used as alternatives to 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.

Acetonitrile

One of the reasons that acetonitrile is so popular is as a result of its very low UV absorbance, even at 220 nm. It gives 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.

The gradient conditions required for elution are as follows:

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

The chromatogram in Figure 1 does show a very small amount of baseline drift, even with acetonitrile, but this is largely due to the sensitivity of the UV at 220 nm.

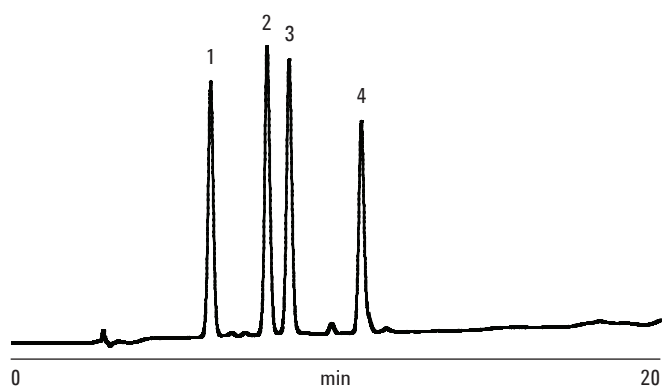


Figure 1. Peptide mixture on PLRP-S 100Å 10 µm 250 x 4.6 mm ID column at 1.0 mL/min. Gradient elution of 0-100% B in 20 minutes. UV detection at 220 nm.

Compounds: 1. Oxytocin, 2. Angiotensin II, 3. Angiotensin I, 4. Insulin.

However, when run on a 385-LC ELS detector, the chromatogram in Figure 2 can be obtained. The ELSD is run with the nebulizer temperature at 40 °C, evaporation temperature at 70 °C, and gas flow to 1.20 SLM. 10 µL is injected as above.

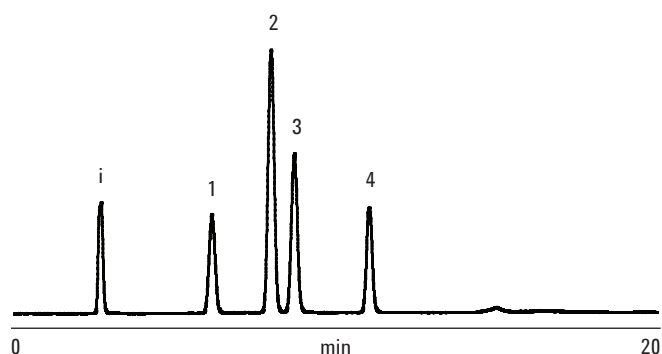


Figure 2. Peptide mixture on PLRP-S 100Å 10 µm 250 x 4.6 mm ID column at 1.0 mL/min. Gradient elution of 0-100% B in 20 minutes and ELS detection.

Compounds: i. Impurity 1. Oxytocin, 2. Angiotensin II, 3. Angiotensin I, 4. Insulin.

As expected, the chromatogram produced by the ELSD has a very flat stable baseline, with very little noise. An additional peak at around 2.8 minutes is shown, however, which could be due to a non-volatile salt that is not visible on the UV detector.

The UV at 220 nm gives a greater response for the peptides, in terms of peak height, than the ELSD but also gives the greatest amount of noise. See Table 1 overleaf.

Table 1. Comparison of peak response on UV and ELS detectors with acetonitrile-based eluents.

	UV at 220 nm		ELSD	
	Peak height (mV)	Peak Area (μV.Min)	Peak height (mV)	Peak Area (μV.Min)
Oxytocin	387	72146	70	15205
Angiotensin II	439	82348	186	41784
Angiotensin I	417	80741	113	25295
Insulin	321	63518	74	16285

Methanol

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.

Mobile phase conditions were modified as below, however methanol gives a very high background absorbance at 220 nm. As a result, the separation also had to be run at 280 nm. This gives the separations shown in Figures 3.

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 20 minutes
 Flow Rate: 1 mL/min
 Temperature: Ambient

Detection: As described

At 220 nm, the high absorbance of the methanol causes a large peak as the gradient moves towards the high percentage organic portion of the gradient (as shown in Figure 3A). This solvent peak actually overwhelms the insulin peak.

As a result, the separation had to be run at 280 nm, which gives a flatter baseline, as shown in Figure 3B. However, this gives much lower absorbance for the four peptides and a greater injection volume is required to obtain peaks of a reasonable size.

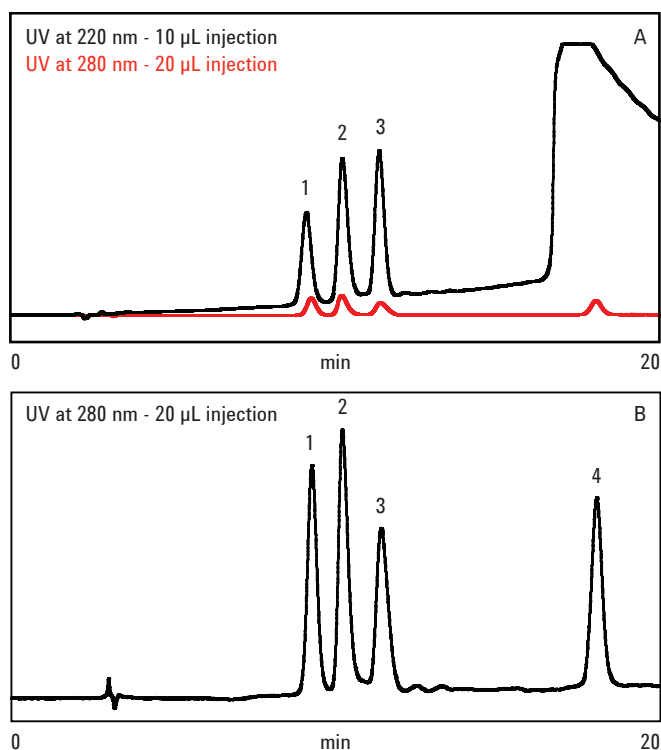


Figure 3. Peptide mixture on PLRP-S 100Å 10 μm 250 x 4.6 mm ID column at 1.0 mL/min. Gradient elution of 40-80% B in 20 minutes. A). Comparison of response with UV detection at 220 nm and 280 nm, B). UV detection at 280 nm. Compounds: 1. Oxytocin, 2. Angiotensin II, 3. Angiotensin I, 4. Insulin.

Table 2. Comparison of peak response on UV and ELS detectors with methanol-based eluents.

	UV at 220 nm		UV at 280 nm		ELSD	
	Peak Height (mV)	Peak area (μV.Min)	Peak Height (mV)	Peak area (μV.Min)	Peak Height (mV)	Peak area (μV.Min)
Oxytocin	331	118079	61	20422	126	38844
Angiotensin II	519	182919	70	22858	223	66460
Angiotensin I	533	182729	42	16704	238	71822
Insulin	-	-	50	18629	241	71000

When run on a 385-LC ELS detector, the chromatogram in Figure 4 can be obtained. The ELSD is run with the nebulizer temperature at 30 °C, evaporation temperature at 30 °C, and gas flow to 1.80 SLM. 10 μL is injected as above.

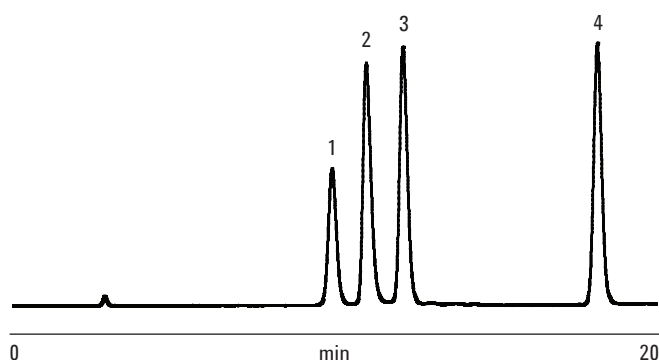


Figure 4. Peptide mixture on PLRP-S 100Å 10 μm 250 x 4.6 mm ID column at 1.0 mL/min. Gradient elution of 40-80% B in 20 minutes and ELS detection. Compounds: 1. Oxytocin, 2. Angiotensin II, 3. Angiotensin I, 4. Insulin.

The difference between the UV and ELS detectors is much more apparent with this mobile phase, as the chromatogram produced by the ELSD has a very flat stable baseline, with very little noise, and no large solvent peak towards the end of the run.

In terms of their relative sensitivities, the UV at 220 nm gives the greatest response in terms of peak height (but the greatest amount of noise), the ELSD is next, followed by the UV at 280 nm (which gives a very low response, even with double the injection volume). As shown in Table 2 above.

Ethanol

Another alternative solvent 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

Injection Volume: 10 μL
Detection: UV at 220 nm

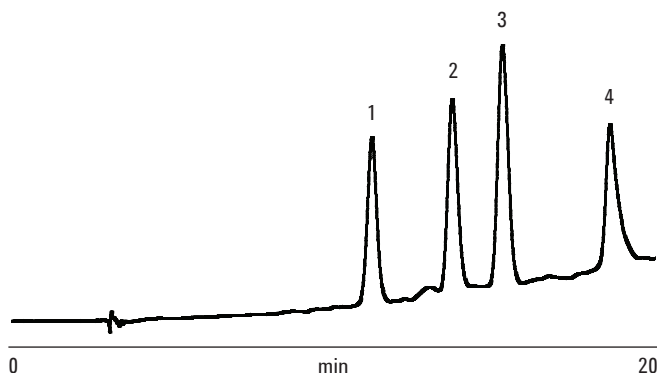


Figure 5. Peptide mixture on PLRP-S 100Å 10 μm 250 x 4.6 mm ID column at 1.0 mL/min. Gradient elution of 20-60% B in 20 minutes. UV detection at 220 nm. Compounds: 1. Oxytocin, 2. Angiotensin II, 3. Angiotensin I, 4. Insulin.

Table 3. Comparison of peak response on UV and ELS detectors with ethanol-based eluents.

	UV at 220 nm		ELSD	
	Peak height (mV)	Peak Area (μV.Min)	Peak height (mV)	Peak Area (μV.Min)
Oxytocin	382	130309	108	30562
Angiotensin II	436	154270	153	44429
Angiotensin I	553	206870	187	63620
Insulin	325	141316	137	60886

Ethanol also appears to have a greater UV absorbance than acetonitrile at 220 nm, as the baseline drift during the gradient is much more significant. However, no large solvent peak elutes at the high percentage organic portion of the gradient, as with methanol. See Figure 5.

However, when run on a 385-LC ELS detector, the chromatogram in Figure 6 can be obtained. The ELSD is run with the nebulizer temperature at 30 °C, evaporation temperature at 30 °C, and gas flow to 1.60 SLM. 10 μL is injected as above.

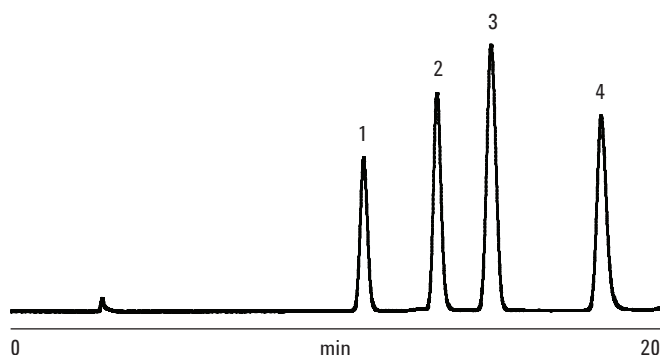


Figure 6. Peptide mixture on PLRP-S 100Å 10 μm 250 x 4.6 mm ID column at 1.0 mL/min. Gradient elution of 20-60% B in 20 minutes, and ELS detection. Compounds: 1. Oxytocin, 2. Angiotensin II, 3. Angiotensin I, 4. Insulin.

Once again, the ELSD gives very flat stable baseline and no background noise. Its sensitivity is slightly less than the UV at 220 nm, see Table 3 above.

Conclusion

These results show two alternative solvents to acetonitrile that can be used for the reversed phase HPLC analysis of peptides.

With the aid of a 385-LC ELS detector, equivalent separations can be obtained with ethanol and methanol based mobile phases under modified gradient elution conditions.

The 385-LC ELSD is capable of evaporating a wide range of different solvents, resulting in completely flat baselines and sharp peaks, even during gradient elution.

The 385-LC incorporates a programmable adjustable gas flow setting, that can be used to counter-act the effect of viscosity changes during gradient elution in order to maintain a uniform detector response. Unlike other ELS detectors, the 385-LC's unique gas control can be used to deliver peak areas that are directly proportional to the concentration irrespective of the mobile phase gradient (i.e. for the samples described in this application note, where each analyte has a concentration of 1 mg/mL), the compounds would have the same peak area across the gradient.

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