

## APPLICATION NOTE

### Automated Digestion of Horse Heart Myoglobin and Horse Skeletal Muscle Myoglobin using the Acquity UPLC I class Plus and the Acquity UPLC® BEH C18 1.7 µm 2.1x50 cm column as the final Reversed Phase Column.

In previous Application Notes the proteins were used without any need for denaturation.

The concentration of buffers did not interfere with complete digestion.

In the present case of Myoglobins, it is important to use buffer of low concentrations such as 10 mM however there is no need to denature the protein.

Myoglobin samples are made in buffer A that contains 2% ACN with a concentration of 10 mg/ml.

Buffer A: 0.1 % TFA in DI H<sub>2</sub>O: ACN 98: 2 (for peptide mapping)

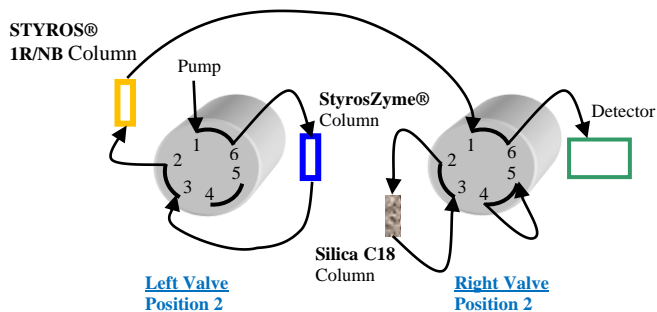
Buffer B: 0.1 % TFA in ACN: H<sub>2</sub>O, 70: 30 (for peptide mapping)

Buffer C: 10 mM TRIS, pH= 8.5 (for digestion).

The columns are:

A Narrow Bore enzyme column of 2.1x50 mm stainless Steel (StyrosZyme® TPCK-Trypsin), with the same size reversed phase Narrow Bore column (STYROS® 1R) ending with the Silica C18 column (Acquity UPLC® BEH C18 1.7 µm 2.1x50 cm column).

The following shows the starting position of the valves used:



**Setup 1**

In this first position, the StyrosZyme® enzyme column and the polymeric STYROS® 1R/NB columns only, are online and the Silica C18 column is not exposed to any high pH aqueous buffer that is used for the digestion.

It is therefore possible to safely scout the right digestion buffer as well as the optimum conditions for the full digestion prior to the use of the silica column.

#### 1-Equilibrate the enzyme column with both columns in line as shown in Setup 1.

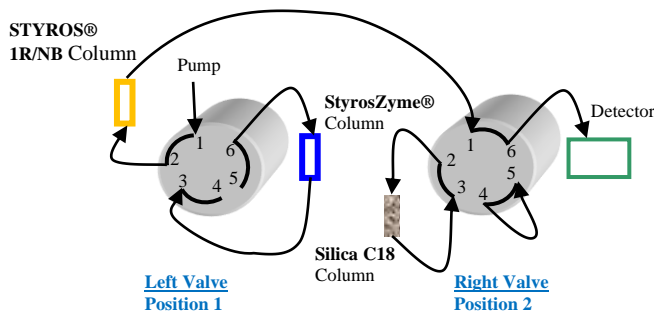
Time	% of buffer C	Flow rate (ml/min)
0		0
0.01	100	0.2
6	100	0.2

#### 2-With both columns in line as in Setup 1, the Myoglobin sample (1 µl) is injected, and the resulting digests are dumped on

#### the polymeric reversed phase column using the following method:

Time	% of buffer C	Flow rate (ml/min)
0		0
1	100	0.2
5	100	0.2

In the second set up, the left valve is switched to position 1 still with only the STYROS® 1R polymeric reversed phase column online in order to equilibrate it with the starting solvent gradient.



**Setup 2**

#### 3-The reversed phase column only is now online as in Setup 2 to wash the leftover salt and prepare it for the reversed phase mapping.

It is also ready for hyphenation with a mass spectrometer.

Time	% of buffer B	% of buffer A	Flow rate (ml/min)
0			0
1	0	100	0.2
15	0	100	0.2

#### 4-The digested peptides are now trapped on the polymeric reversed phase column and can be mapped following a gradient.

The setup is now as Setup 2.

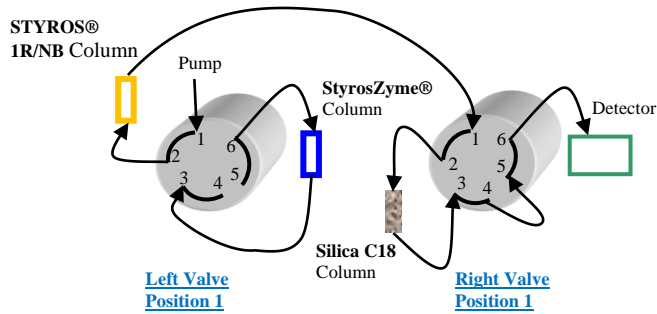
Time	% of buffer B	% of buffer A	Flow rate (ml/min)
0			0
0.01	0	100	0.2
20	60	40	0.2

When the conditions of the digestion are satisfactory, one can use the second switching valve to bring the Silica C18 column online as shown in Setup 3.

The same gradient as in step 4 can be used for the mapping to run the separation on the C18 Silica column:

**4'-The digested peptides trapped on the polymeric reversed phase column can now be mapped on the C18 Silica column. The setup is now as Setup 3.**

Time	% of buffer B	% of buffer A	Flow rate (ml/min)
0			0
0.01	0	100	0.2
20	60	40	0.2



### Setup 3

To re-equilibrate both reversed phase columns that are now online, a similar step than step 5 is used.

**5- Both reversed phase columns are pre-equilibrated to the initial low organic prior to getting in contact with the digestion buffer. The setup is now Setup 3.**

Time	% of buffer B	% of buffer A	Flow rate ml/min
0			0
1	0	100	0.2
15	0	100	0.2

**6-In the final step the line and the reversed phase column are flushed clean from any leftover organic going to the enzyme column, still avoiding the Silica column. The setup therefore remains as setup 2.**

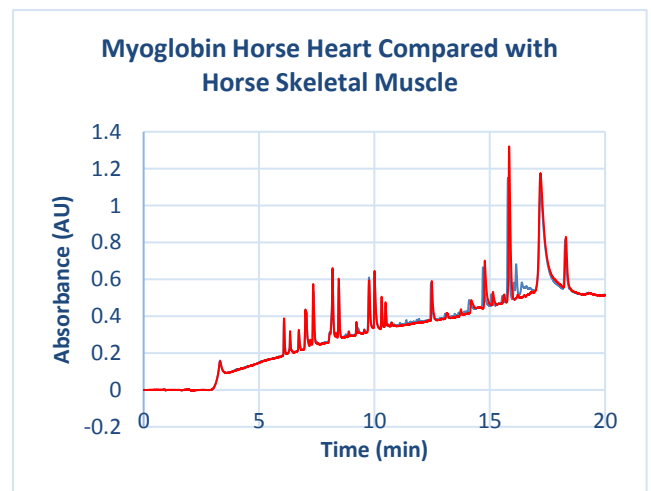
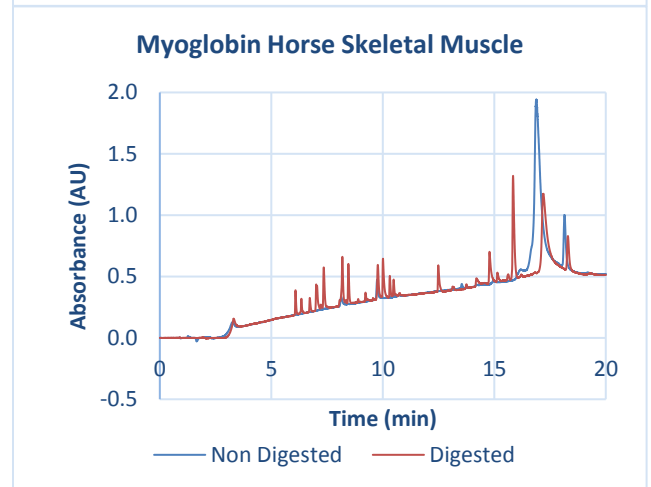
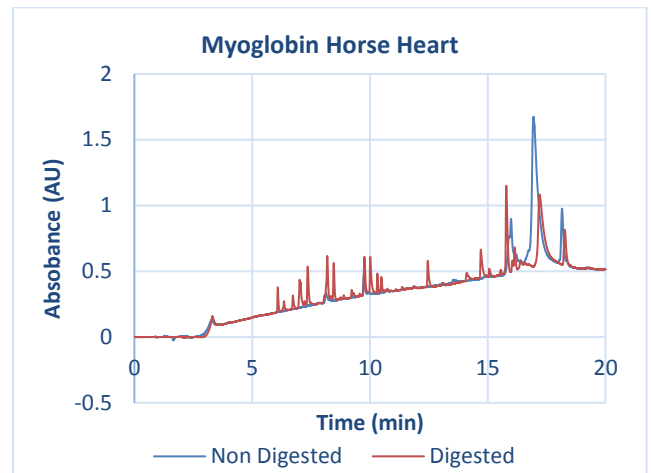
Time	% of digestion buffer C	Flow rate (ml/min)
0		0
0.01	100	0.2
6	100	0.2

The system is now ready for the next cycle to check the reproducibility of the digestion.

The use of Narrow Bore columns requires minimal use of solvents therefore the automated digestion and mapping can be run around the clock without any concern of running out of buffers or overfilling the waste.

The temperature is set at 37°C for all sequences.

The following chromatograms are the results of this process and show the variations between the two Myoglobins.



Fully automated using a non-leaching Simulated-Monolith™ matrix stable at all pH's.

