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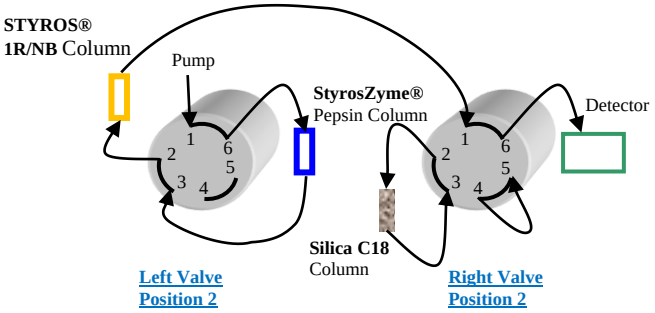
APPLICATION NOTE

Automated StyrosZyme® Pepsin digestion of Myoglobin from horse heart and horse skeletal muscle, with Immobilized Enzyme on Polymeric Hard Gel Simulated-Monolith™ Enzyme Reactor with the Acquity UPLC *I* class Plus and Final Silica C18 mapping

Using Pepsin, an acidic protein with a pI of 1.0, we have digested online and in a fully automated procedure, 2 Myoglobins from horse heart and horse skeletal muscles.
Below pH 6, pepsin preferentially cleaves on the carboxyl side of L-Phenylalanine, L-Leucine, or L-Tyrosine, where the amino side residue is preferably, but not limited to, an amino acid containing a hydrophobic side chain.
The results were compared with each other in the absence of autodigestion of the enzyme that has retained its full digestive capabilities throughout the process.

The buffers used are.
Buffer A: 0.1 % TFA in DI H2O: ACN 98:2 (for peptide mapping)
Buffer B: 0.1 % TFA in ACN: H2O, 70:30 (for peptide mapping)
Buffer C: 0.1 M Phosphate, pH= 2.5 (for digestion).
The 3 columns used are all narrow bore, therefore minimizing high volumes of solvent as well as avoiding the generation of large volume of waste
An Enzyme column of 2.1x100 mm stainless Steel (StyrosZyme® Pepsin).
Two narrows bore reversed phase columns. One polymeric (STYROS® 1R) with high capacity and high pH tolerance and one Silica C18 column (Acquity UPLC® BEH C18 1.7 µm 2.1x50 cm column) with high performance.
The following shows the starting position of the valves used:
The UPLC *I* class Plus is fitted with 2 six port valves.
The following shows the starting position of the valves used:



Setup 1

In this first position, the StyrosZyme® Pepsin column and the polymeric STYROS® 1R/NB column only, are online and the Silica C18 column is not exposed to any excessive pH 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.
Both polymeric columns, StyrosZyme® TPCK-Trypsin and STYROS® R reversed phase polymeric can tolerate extreme pH's.

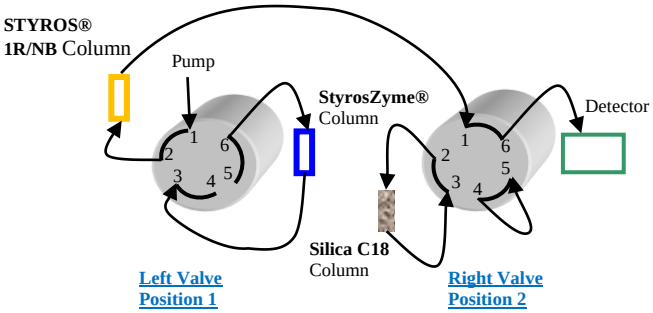
1-Equilibrate the enzyme column and the polymeric column with the digestion buffer, in line, as shown in Setup 1.

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, 5 µl of a solution of 5 mg/ml protein in buffer A is injected, and the resulting digests are dumped on the reversed phase polymeric column using the following method:

Time	% of buffer C	Flow rate (ml/min)
0		0
0.01	100	0.2
10	100	0.2

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



Setup 2

3-The reversed phase Polymeric column only is now in line 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	0.3
5	0	100	0.3

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.

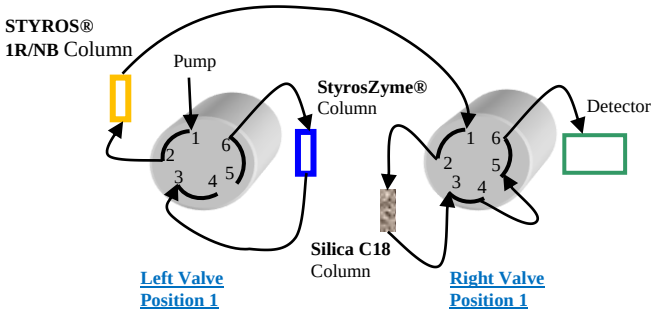
Time	% of buffer B	% of buffer A	Flow rate (ml/min)
0			0
0.01	0	100	0.2
20	85	15	0.2

Once 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 in addition:

4'-The digested peptides trapped on the polymeric reversed phase column can now be mapped on the C18 Silica column in addition.

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	85	15	0.2



Setup 3

4''-In this step, using the same setup 3, the columns are washed with high organic to make sure they are devoid of any leftover proteinaceous species.
The setup remains Setup 3.

Time	% of buffer B	% of buffer A	Flow rate (ml/min)
0			0
1	60	40	0.3
2	100	0	0.3
4	0	100	0.3
8	0	100	0.3

To re-equilibrate both reversed phase columns that are now in line, the following step 5 is used.

5- Both reversed phase columns are preequilibrated 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	0.3
10	0	100	0.3

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

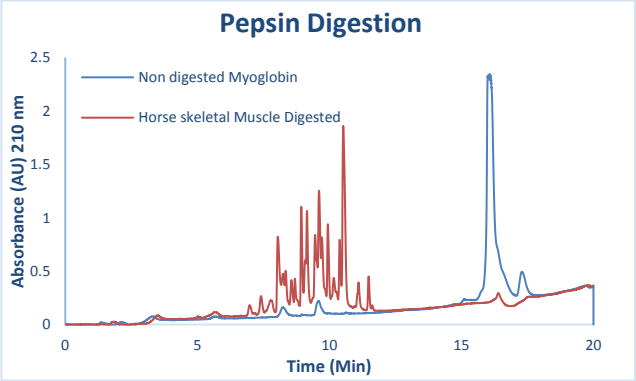
We have used 5 µl of a solution of 5 mg/ml of protein in buffer A as sample to digest.

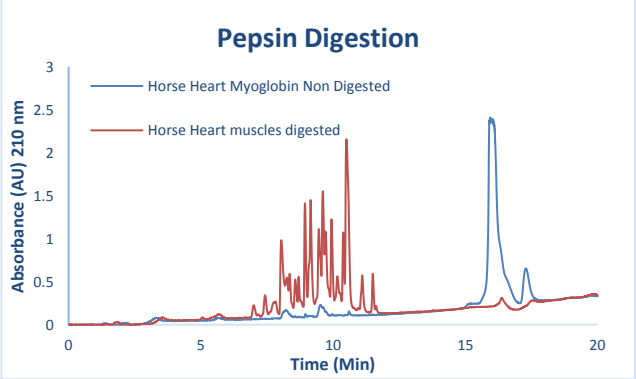
The temperature is set at 37°C for all sequences.
The digestion is monitored at 210 nm primarily.
They are done under similar conditions
The enzyme column of 2.1 x 100 mm has a volume to 0.346 ml. A total of 2 ml is run through it during the digestion at a volumetric flow rate of 0.2 that is a velocity of 374 cm/hr calculated on an empty column.

Each digest is compared with the same amount of corresponding starting intact protein (5µl).

The performance of the STYROS® 1R polymeric is crucial in the final separation of the digested peptides,

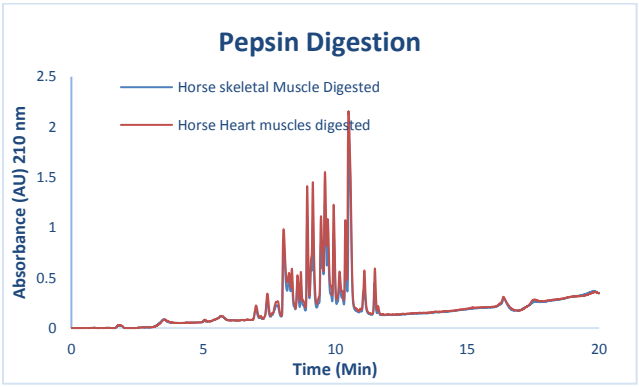
Same amount of protein (5 µl) is injected under the same conditions and the setup 3 during a 20-minute gradient run.
The amount injected is clearly saturating the narrow bore polymeric and silica columns. This is the amount of protein that is run through the column and is fully digested.





The chromatograms of the digestions are compared with the original nondigested sample that was injected.
As it shows the digestion is complete under the present automated conditions. It is also reproducible during several injections indicating the extent of the enzyme column stability.

The following superimposed chromatograms compares the digests of the two types of Myoglobins with StyrosZyme® Pepsin



Minimal differences are noticed.