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APPLICATION NOTE

Automated digestion of Cytochrome c from bovine compared with Equine with StyrosZyme® TPCK-Trypsin, Immobilized Enzyme on Polymeric Hard Gel Simulated-Monolith™ Enzyme Reactor with the Acquity UPLC *I* class Plus and Final Silica C18 mapping.

Trypsin is a medium size globular pancreatic serine protease that cleaves protein bounds on the C terminal of the amino acid residues lysine and arginine.
The optimum pH for Trypsin is 9 and the optimum temperature is 65°C. However, these numbers hold true when the digestion proceeds in batch.

In the case of online digestion, we were able to achieve full digestion of the proteins (two types of Cytochrome c) in full at 37°C and pH of 8.5 using 0.1 M Tris buffer at pH=8.5

The tandem reversed phase column in such set up needs to tolerate high pH's.
Polymeric reversed phase columns such as STYROS® 1**R and 2R** are highly recommended, especially when the effluents are used to feed a mass spectrometer.

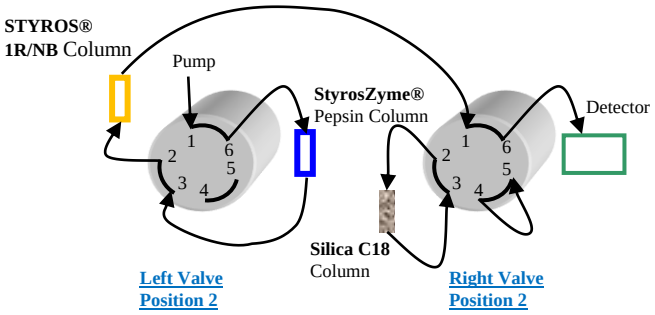
The presence of some denaturing agents such as organic solvents, TFA or 3M guanidine. The proteins that are difficult to digest in solution will be difficult to digest online. It is necessary to assist the unfolding of the protein by denaturing agents and prevent the refolding by using reducing or alkylating agents.

We have considered two types of Cytochrome c from Equine and from Bovine to run the digestion online with StyrosZyme® TPCK-Trypsin.

The 3 columns used are all narrow bore, therefore minimizing high solvent consumption as well as the generation of waste
The Enzyme column used is a 2.1x100 mm stainless Steel (StyrosZyme® TPCK-Trypsin).
Two narrow bore reversed phase columns. One polymeric (STYROS® 1R) with high capacity, high pH tolerance and high performance (2.1x50 cm) column as well as a Silica C18 column (Acquity UPLC® BEH C18 1.7 µm 2.1x50 cm column) with high performance.

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 Tris pH= 8.5(for digestion).

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® TPCK-Trypsin 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 high pH.

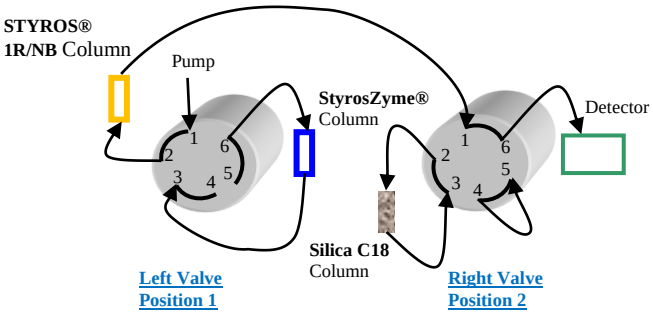
1-Equilibrate the enzyme column with all columns except the silica column, in line, as shown in Setup 1.

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, 3 µl of a solution of 10 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	0.1
2	100	0.3
5	100	0.3

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
0.01	0	100	0.3
10	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.

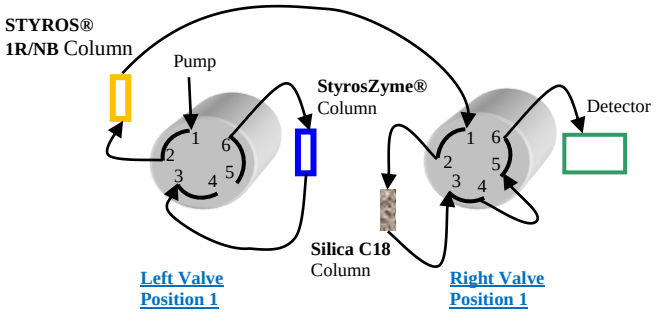
Time	% of buffer B	% of buffer A	Flow rate (ml/min)
0			0
0.01	0	100	0.2
20	70	30	0.2

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	70	30	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
0.01	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
0.01	0	100	0.3
10	0	100	0.3

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	0.3
6	100	0.3

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

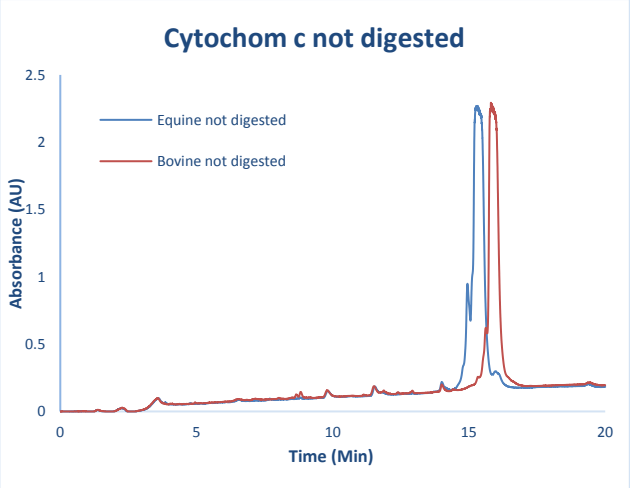
We have used 3 µl of a solution of 10 mg/ml of protein in buffer A as sample to digest.

The temperature is set at 37°C for all sequences.
We have compared Cytochrome c from Equine with Cytochrome c from Bovine and noticed the differences.

The digestions are done under similar conditions
The enzyme column of 2.1 x 100 mm has a volume to 0.346 ml. A total of 1.3 ml is run through it during the digestion at a volumetric flow rate of 0.1 to 0.3 ml/min.

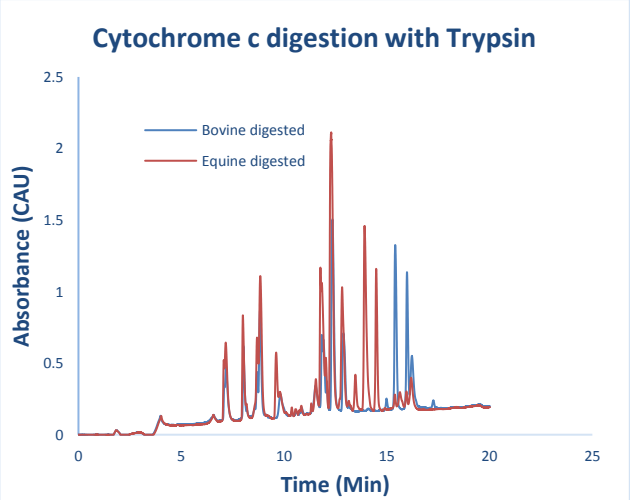
Each digest is compared with the same amount of corresponding starting intact protein (3µl).

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



Same amount of protein (3 µl) is injected under the same conditions and the setup 3 during a 20 minutes 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 following superimposed chromatograms show the digests of the two types of Cytochrome c with StyrosZyme® TPCK-Trypsin



Each of the two Cytochrome c is also compared with the original nondigested sample that was injected.
As is shows the digestion is complete under the present automated conditions. It is also reproducible during a number of injections.