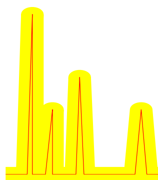

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APPLICATION NOTE

Comparing Tryptic digest from Bovine with Porcine, using StyrosZyme® TPCK-Trypsin, Immobilized Enzyme on Polymeric Hard Gel Simulated-Monolith™ Enzyme Reactor with the Acquity UPLC *I* class Plus and Final Silica mapping.
Update of Application Notes 153 and 170.

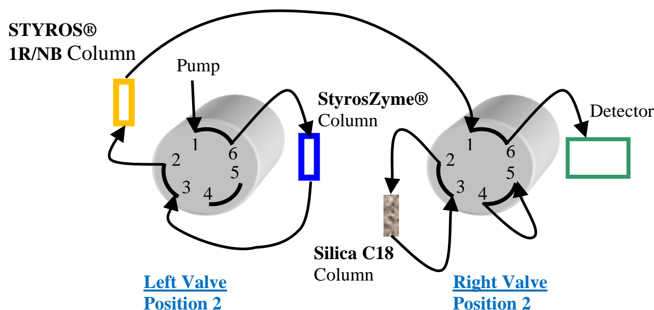
Commercial trypsin is usually isolated from porcine pancreas. It carries the risks of infectivity and immunogenicity.
To prevent such risks and avoid additional steps in the manufacture of biopharmaceuticals during purification, vaccines in particular, the solution is to immobilize the enzyme and avoid its presence in the mixtures therefore avoid additional steps of removing it again from the mixture even in early stages of production to avoid compliancy with regulations.

However, it requires the use of a stable non leaching and high pH tolerant media.
No manufacturer to date can provide such product. The industry has therefore moved in resorting to recombinant alternative that are costly and have new issues, high autolysis rates among others.
OraChrom has succeeded in the manufacture of stable Styrene-Divinyl-Benzene polymers with active hydrophilic coating as a stable base for the immobilization.
As proof of stability, several enzymes have been immobilized and tested.
The present Application Note is an additional one that compares the digestion of bovine Trypsin to porcine Trypsin by using StyrosZyme® TPCK-Trypsin from bovine.

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= 8.55 (for digestion).

3 columns are also used:
A conventional bore enzyme column of 4.6x50 mm stainless Steel (StyrosZyme® TPCK-Trypsin). A reversed phase polymeric Narrow Bore column (STYROS® 1R) of 2.1 x 50 mm, 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 column 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.
Both polymeric columns can tolerate high pH.

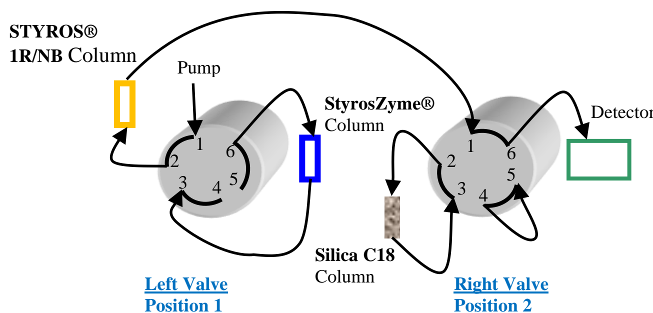
1-Equilibrate the enzyme column with all columns except the silica column, in line, as shown in Setup 1.

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

2-With both columns in line as in Setup 1, 10 µl of a solution of 10 mg/ml Trypsin in buffer A is injected in the conventional bore column with a linear velocity of ~70 cm/hr and the resulting digests are dumped on the reversed phase column using the following method:

Time	% of buffer C	Flow rate (ml/min)
0	100	0.2
1	100	0.2
10	100	0.2

In the second setup, 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	5	95	0.2
1	5	95	0.2
10	5	95	0.2

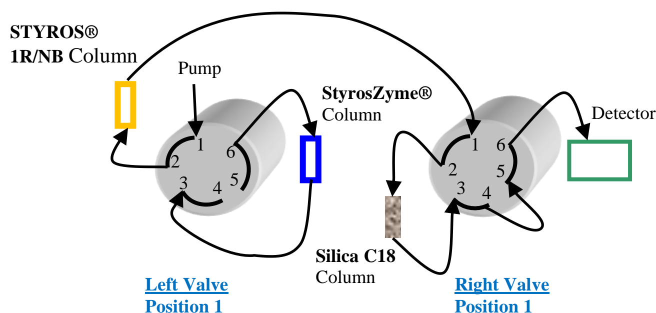
4-The digested peptides are now trapped on the polymeric reversed phase column and can be mapped following a gradient.
The setup remains as Setup 2.

Time	% of buffer B	% of buffer A	Flow rate (ml/min)
0	5	95	0.2
0.01	5	95	0.2
90	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	5	95	0.2
0.01	5	95	0.2
90	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	5	95	0.2
1	100	0	0.2
4	100	0	0.2
4.1	5	95	0.2
8	5	95	0.2

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 additionally 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	5	95	0.2
0.01	5	95	0.2
5	5	95	0.2

The system is now ready for the next cycle to check the reproducibility of the digestion.
The temperature is set at 37°C for all sequences. The absorbance is monitored by the PDA at 214 nm.

The digestions are complete in both cases, they are reproducible and there is no sign of deactivation of the column's immobilized Trypsin.
However, the digestions profiles are drastically different.

While Trypsin from Bovine can be fully digested without the addition of DTT (1,4-Dithiothreitol), to fully digest Trypsin from Porcine, DTT is necessary.