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APPLICATION NOTE

Testing StyrosZyme® TPCK-Trypsin Column's Activity Using Insulin B Chain. Fully Automated Using Waters Acquity UPLC *I* class Plus.

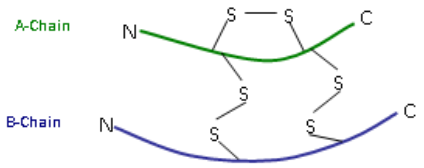
It is important for the end user to test the activity of the enzyme reactor column in a regular basis.
In the present application note we have used a 2.1 x 50 mm Stainless Steel StyrosZyme® TPCK-Trypsin Simulated-Monolith™ column that was used for over 2,000 injections during the past 6 months coupled with a STYROS® R reversed phase column Simulated-Monolith™ as well and of similar size and finally a Silica C18 column (Acquity UPLC® BEH C18 1.7 µm 2.1x50 cm column).

The use of the Waters Acquity UPLC *I* class Plus has shown to be the proper instrument for the automation of the process.

The sample is made of oxidized insulin B Chain with a concentration of 3 mg/ml.
The 3 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: 100 mM TRIS, 100 mM NaCl, pH= 8.5 (for digestion).

Oxidized Insulin B Chain is one of the two chains forming the active form of insulin:



It consists of the following amino-acid sequence:

Phe-Val-Asn-Gln-His-Leu-Cys-(SO3H)-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys-(SO3H)-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala

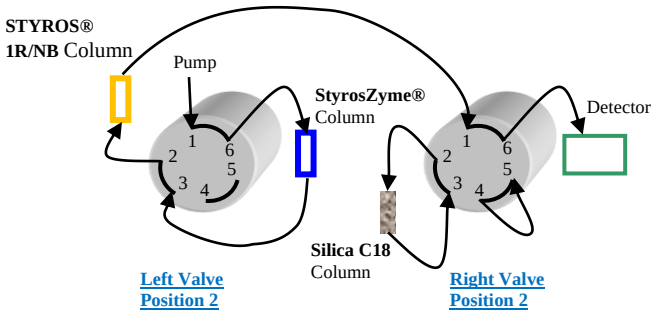
A typical digestion of insulin in solution with high grade Trypsin requires at least 18 hours at 37 °C.

StyrosZyme® TPCK-Trypsin can achieve the digestion online at 37°C, in 10 minutes at 0.05 ml/min. That is a linear velocity of close to 87 cm/hr. based on an empty column.

The digestion and mapping proceeds in under an hour with a 2.1 x 50 mm enzyme reactor column.
The resulting peptides can be mapped directly after digestion by using a STYROS®1R reversed phase column connected in series to the immobilized enzyme column in10 minutes.

The mapping can further be enhanced using a silica column in series with the reversed phase polymeric.

The following schematics show clearly the process by using two switching 6 port valves set on the Acquity UPLC *I* class Plus.



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.

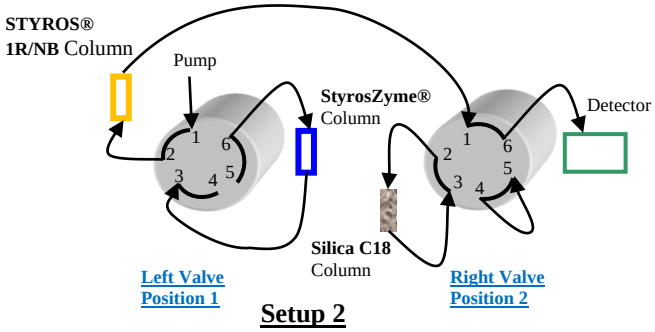
1-Equilibrate the enzyme column with both columns in line as shown in Setup 1.

Time	% of buffer C	Flow rate (ml/min)
0		0
0.01	100	0.2
2	100	0.2

2-With both columns in line as in Setup 1, Insulin 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	0.05
10	100	0.05

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
10	0	100	0.2

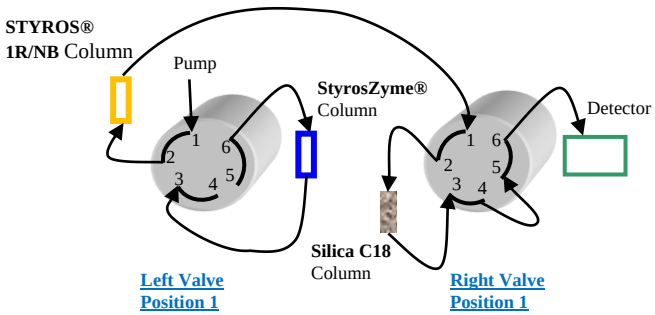
4-The digested peptides are now trapped on the polymeric reversed phase column and can be mapped following a gradient.
The setup is now as Setup 2.

Time	% of buffer B	% of buffer A	Flow rate (ml/min)
0			0
0.01	0	100	0.2
10	100	0	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 both the polymeric and the C18 Silica column.

4'-The digested peptides trapped on the polymeric reversed phase column can now be mapped on the added 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
10	100	0	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
6	0	100	0.2

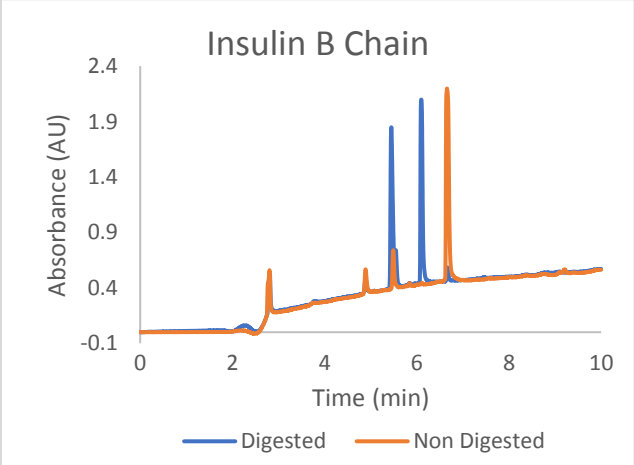
6-In the final step the line and the reversed phase column are flushed clean from any leftover organic going to the enzyme column, still avoiding the Silica column. The setup therefore remains as setup 2.

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



This allows the end user to periodically test the column's activity during multiple overnight runs as well as its reproducibility.