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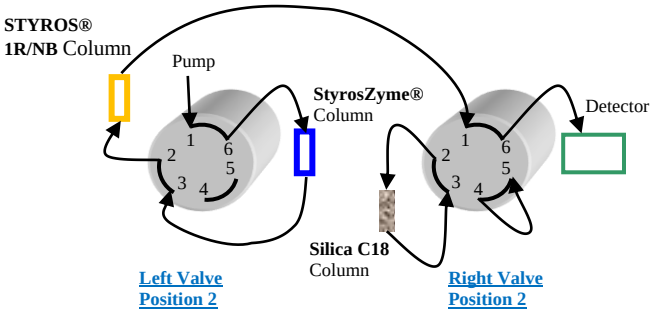
APPLICATION NOTE

Adding DTT to Fully Digest Trypsin Inhibitor with Immobilized TPCK-Trypsin. Case study of digestion with StyrosZyme® TPCK-Trypsin Hard Gel Simulated-Monolith™ Enzyme Reactor with the Acquity UPLC *I* class Plus and Final Silica C18 mapping.

Using the same established setups as the previous Application Notes for online digestions, we have explored using Trypsin Inhibitor from chicken, to study the possibility to digest such proteins without the need to denature or alkylate them.
The stability of the polymeric base matrix plays a major role as the stability of the immobilized enzyme that results from it makes it possible to have many injections to be a reliable base for comparison of the results seen.
Such is not the case with unstable soft gel such as agarose and similar media or even polymeric leaching and unstable media. Application Notes 142 and 153, show that the enzyme column remains stable, and it is not affected during the digestion of the Trypsin itself in solution. That it is a controlled digestion of the enzyme in solution and not the immobilized enzyme of the reactor.

Using normal bore columns of 4.6 mm ID, we have increased the interaction time of the immobilized enzyme with the substate to increase the digestion process.

A volumetric flow rate of 0.05 ml/min is the equivalent to 18 cm/hr. of linear velocity with a 4.6 mm diameter column giving the substate ample time during an 8 min run to fully interact with the immobilized and stable enzyme with no risk of autolysis.
To keep in mind that these numbers are for empty columns and increase substantially when the column is packed resulting in higher linear velocities.
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 (buffers with high pH of 8 to 12 are used for such digestions)
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.

The buffers used are as follow:
Buffer A: 0.1 % TFA in H2O: ACN 98:2 (for mapping)
Buffer B: 0.1 % TFA in H2O: ACN 5:95 (for mapping)
Buffer C : 0.1 M TRIS, pH=8.2 for digestion.

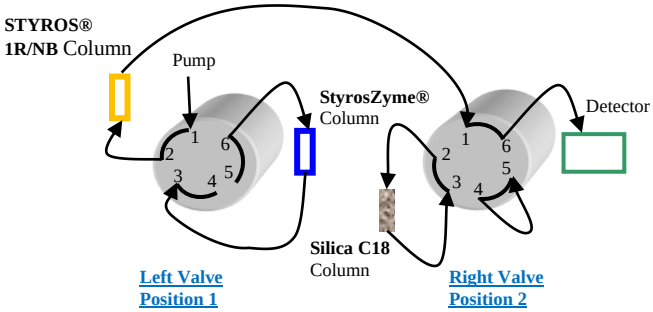
1-Equilibrate the enzyme column with all columns except the silica column, in line, as shown in Setup 1.

Time	% Of buffer C	Flow rate (ml/min)
0	100	0.2
0.01	100	0.2
5	100	0.2

2-With both columns in line as in Setup 1, 10 µl of a solution of 5 mg/ml Trypsin Inhibitor from chicken in buffer A and treated with DTT for 1 hour, 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)
a0	100	0.05
1	100	0.05
8	100	0.05
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 gradient solvent.



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

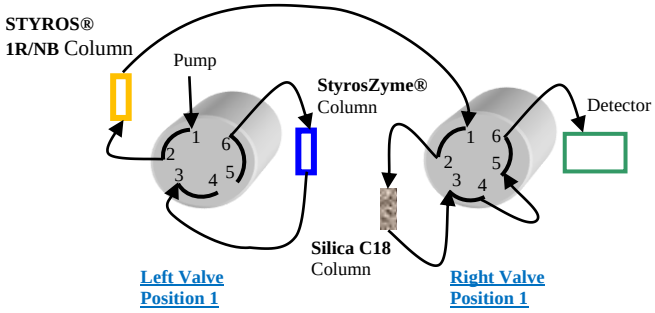
4-The digested peptides are now trapped on the polymeric reversed phase column and can be mapped following a gradient.
The setup remains Setup 2.

Time	% of buffer B	% of buffer A	Flow rate (ml/min)
0	5	95	0.2
0.1	5	95	0.2
60	65	35	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
0.1	5	95	0.2
60	65	35	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.

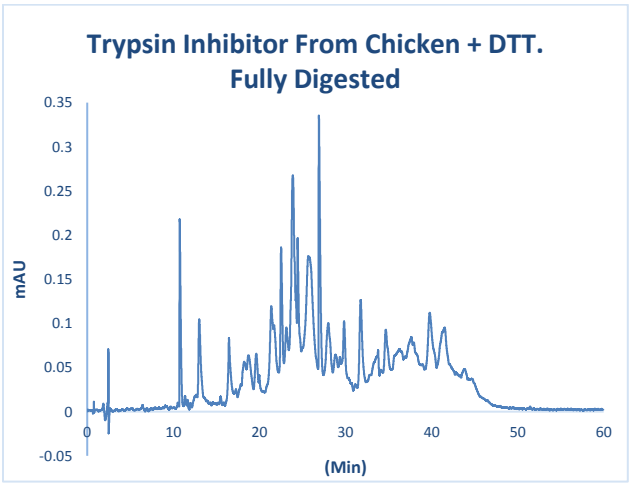
Time	% of buffer B	% of buffer A	Flow rate (ml/min)
0			0
0.01	60	40	0.2
1	100	0	0.2
2	100	0	0.2
4	5	95	0.2
8	5	95	0.2

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

5- In a final step both reversed phase columns are preequilibrated to the initial low organic.
The setup remains setup 3.

Time	% of buffer B	% of buffer A	Flow rate ml/min
0			0
0.01	5	95	0.2
10	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 above chromatogram shows the full digestion of Trypsin Inhibitor from chicken treated with DTT for less than an hour at room temperature.

Inc.