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APPLICATION NOTE

Transferrin. Impact of residency time on the extent of digestion. Case study of digestion of Transferrin with StyrosZyme® TPCK-Trypsin Hard Gel Simulated-Monolith™ Enzyme Reactor with the Acquity UPLC *I* class Plus and Final Silica C18 mapping.

Using similar setups as the previous Application Notes for online digestions, we have explored using Transferrin from human serum, to study this aspect of online and automated digestion.
As the peptides generated are limited and the digestion does not go to completion, the impact of residency time is more noticeable.

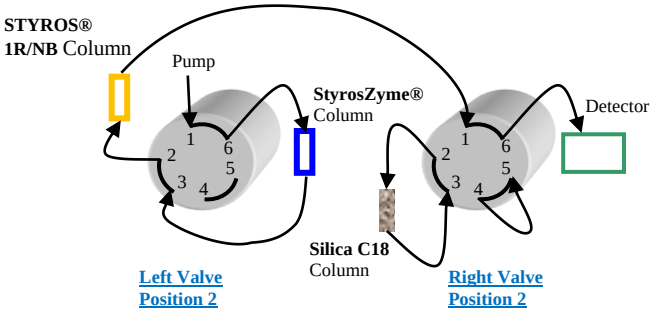
The stability of the polymeric base matrix plays a major role as the stability of the immobilized enzyme that results from it makes it possible to have many injections to be a reliable base for comparison of the results seen.
Such is not the case with unstable soft gel such as agarose and similar or 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.

Columns of different internal diameter, provide the control over the linear velocities and therefore the rate at which the substate protein interacts with the immobilized enzyme.

A volumetric flow rate of 0.2 ml/min is the equivalent to 72 cm/hr. of linear velocity with a 4.6 mm diameter column whereas it increases to 347 cm/hr. for a narrow bore column of 2.1 mm ID.
To keep in mind that these numbers are for empty columns and increase substantially when the column is packed resulting in far higher linear velocities.

During our study, we have used the same volumetric flow rate of 0.2 and 0.05 ml/min for both columns keeping in mind that a narrow bore columns of 2.1 x 50 mm column has a volume of 0.173 ml as compared with a normal bore column of 4.6 x 50 mm with a volume of 0.83 ml. Same observation that these numbers correspond to empty columns and should only be used as a reference of scale.

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.

The buffers used are as follow:
Buffer A: 0.8 % TFA in H2O: ACN 98:2 (for mapping)
Buffer B: 0.8 % TFA in H2O: ACN 70:30 (for mapping)
Buffer C: 0.1 M CO3HNH4, pH=8.2

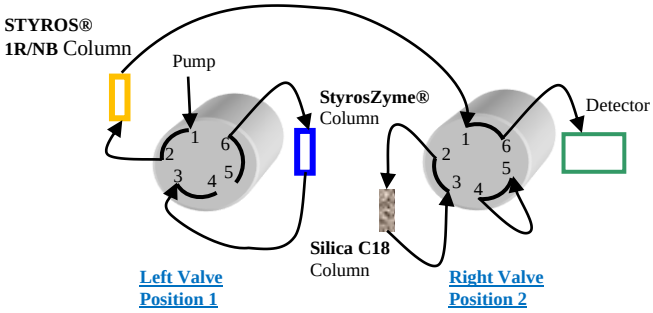
1-Equilibrate the enzyme column with all columns except the silica column, in line, as shown in Setup 1.

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, 1 µl of a solution of 10 mg/ml Transferrin in buffer A is injected, 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
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			0
1	5	95	0.2
10	5	95	0.2

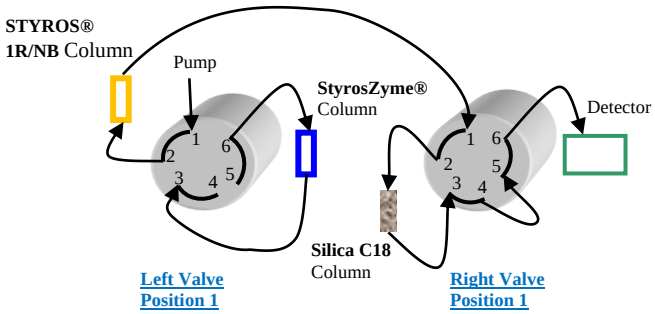
4-The digested peptides are now trapped on the polymeric reversed phase column and can be mapped following a gradient.
The setup is now as Setup 2.

Time	% of buffer B	% of buffer A	Flow rate (ml/min)
0			0
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.

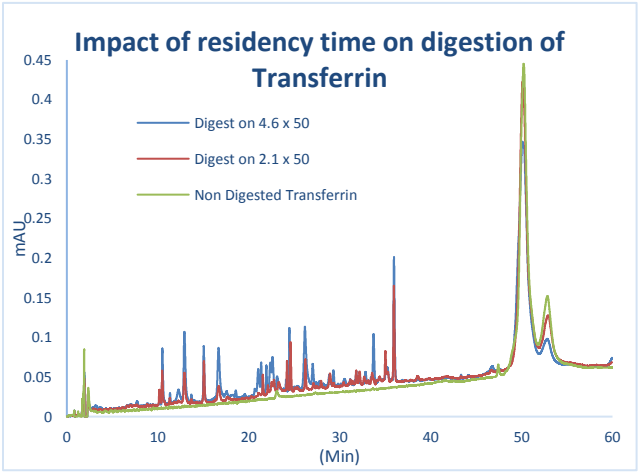
5- Both reversed phase columns are preequilibrated to the initial low organic prior to getting in contact with the digestion buffer.
The setup is now Setup 3.

Time	% of buffer B	% of buffer A	Flow rate ml/min
0			0
0.01	5	95	0.2
10	5	95	0.2

6-In the final step the line and the reversed phase polymeric 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
6	100	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 overlayed chromatograms compare the variability in the peptides generated and the clear difference impacted by the residency time.

The digestion of Transferrin does not go to completion, the small amount of digested protein does make the impact of it clear though.

Inc.