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APPLICATION NOTE

Impact of residency time on the extent of digestion.
Case study of digestion of Human Hemoglobin 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 Hemoglobin from human serum, to study this aspect of online and automated digestion.

It would therefore allow us to realize the extent of control one has over the digestion process as opposed to batch or similar mode of digestions not to overlook or underestimate the stability of the polymeric base matrix itself.

We have shown so far, in Application Notes 142 and 153, that the enzyme column remains stable, and it is not affected during the digestion of the Trypsin itself in solution. That is now a controlled digestion of the enzyme in solution and not the immobilized enzyme of the enzyme reactor.

Using columns of different internal diameter, it would be possible to control the linear velocities or the rate at which the substrate protein interacts with the immobilized enzyme.
A volumetric flow rate of 0.2 ml/min is the equivalent of 72 cm/hr. using 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 increases 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 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:
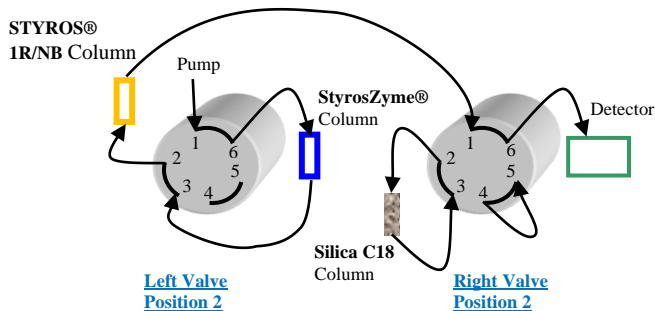
Setup 1

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 buffer 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 Tris, pH = 8.5 (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, 5 µl of a solution of 10 mg/ml Myoglobin 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 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 gradient solvent.
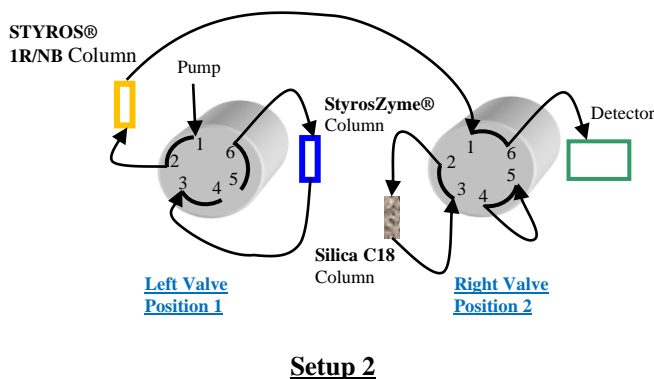
Setup 2

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.

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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.

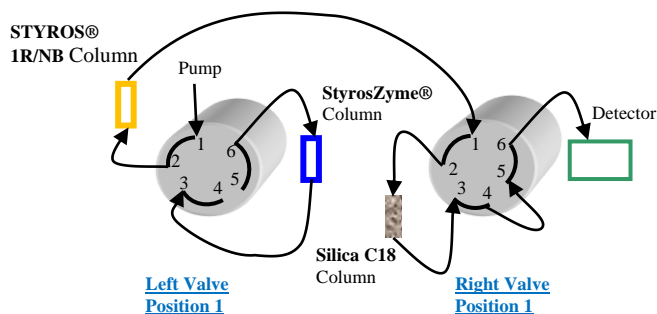| Time	% of buffer B	% of buffer A	Flow rate (ml/min)
0			0
0.1	5	95	0.2
60	100	0	0.2

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	100	0	0.2

STYROS® 1R/NB Column   Pump   StyrosZyme® Column   Detector

Silica C18 Column

Left Valve Position 1   **Right Valve Position 1**

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.

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.
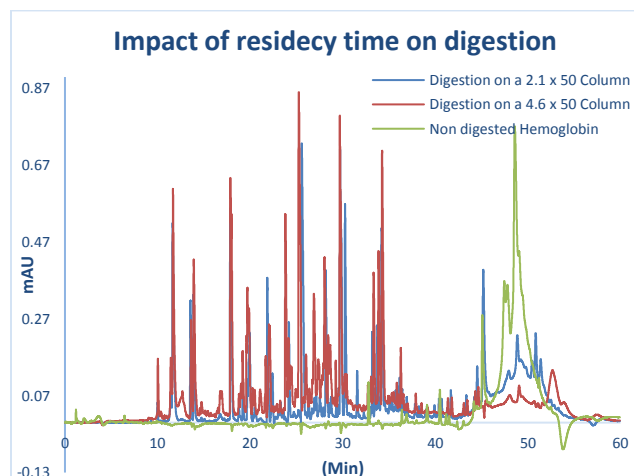**The setup is now Setup 3.**

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 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.

Impact of residecy time on digestion

— Digestion on a 2.1 x 50 Column
— Digestion on a 4.6 x 50 Column
— Non digested Hemoglobin

Note the volumetric flow rate of 0.05 during the first 8 minutes of the digestion for both the Narrow Bore column of 2.1 mm ID as well as the Normal Bore column of 4.6 mm ID.
They respectively correspond to 87 cm/hr for the Narrow Bore column and 18 cm/hr for the Normal Bore column after which the volumetric flow rate increases to 0.2 ml/min during a 2-minute period.
The above overlayed chromatograms compare the extent of digestion and show a clear difference as to the extent of digestion resulting from the residency time of the substate and its interaction with the immobilized enzyme.
Notice the trace of the non-digested Hemoglobin to be compared to both cases to realize that even at succinct interaction with the immobilized enzyme, the digestion is advanced however not as complete as it is with additional residency time in the column.

Inc.