OraChrom, Inc.
The Vanguard of Liquid Chromatography.

10-B Henshaw Street Woburn, MA 01801 USA Phone (781) 932 0151 Fax (781) 932 0787
E-mail: *info@orachrom.com* *www.orachrom.com*

APPLICATION NOTE

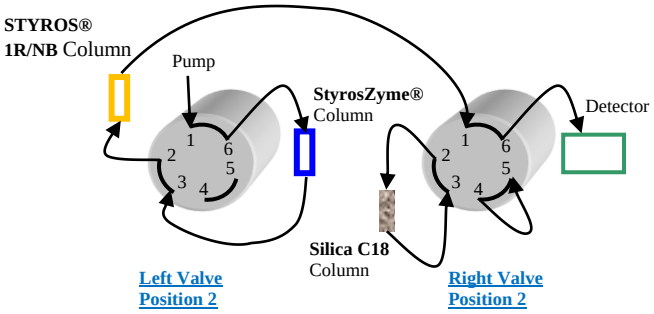
Automated Digestion of Trypsin with StyrosZyme® TPCK-Trypsin, Immobilized Enzyme on Polymeric Hard Gel Simulated-Monolith™ Enzyme Reactor with the Acquity UPLC *I* class Plus and Final Silica mapping. Update of Application Note 153.

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

In this Application Note, similar to AN 153 we have increased the amount of Trypsin sample injected to find out if that would deteriorate the column's activity by digesting the immobilized enzyme.
Same buffers are used.
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).

We used a variation of the 3 columns used before with the enzyme column remaining the same: Narrow Bore of 2.1x50 mm stainless Steel (StyrosZyme® TPCK-Trypsin). The reversed phase polymeric Narrow Bore column (STYROS® 1R) is twice as long (2.1 x 100 mm) ending with the Silica C18 column (Acquity UPLC® HSS C18 1.8 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.

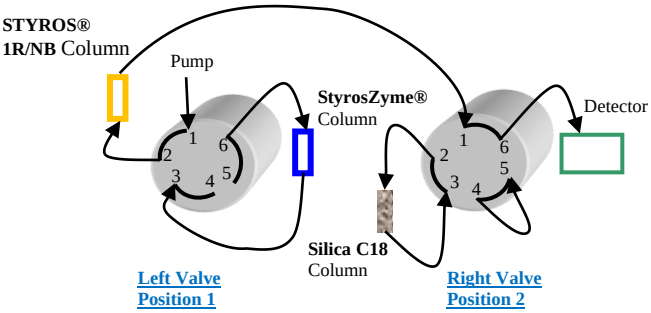
1-Equilibrate the enzyme column with all columns except the silica column, in line, as shown in Setup 1.

Time	% of buffer C	Flow rate (ml/min)
0	100	0.2
1	100	0.2
6	100	0.2

2-With both columns in line as in Setup 1, 0.5, 1 and 10 µl of a solution of 10 mg/ml Trypsin 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		0
1	100	0.1
10	100	0.1

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 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			0
1	0	100	0.2
10	0	100	0.2

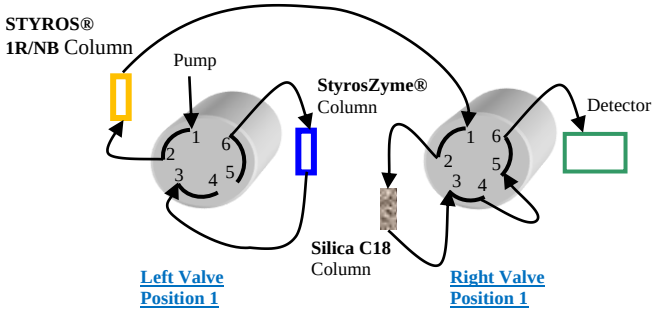
4-The digested peptides are now trapped on the polymeric reversed phase column and can be mapped following a gradient.
The setup is now as Setup 2.

Time	% of buffer B	% of buffer A	Flow rate (ml/min)
0			0
0.01	0	100	0.2
25	60	40	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
0.01	0	100	0.2
25	60	40	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	0	100	0.2
8	0	100	0.

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

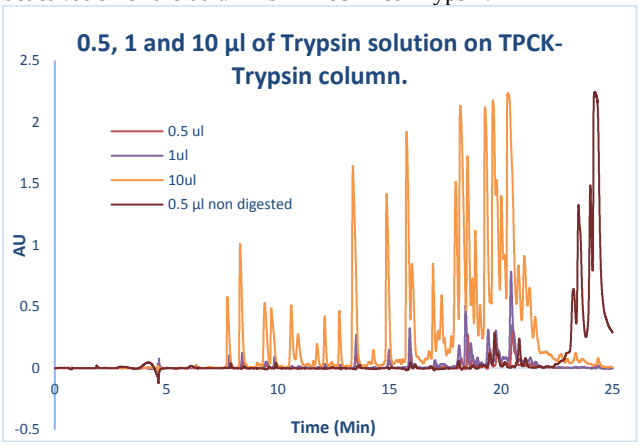
5- Both reversed phase columns are preequilibrated to the initial low organic prior to getting in contact with the digestion buffer.
The setup is now Setup 3.

Time	% of buffer B	% of buffer A	Flow rate ml/min
0			0
0.01	0	100	0.2
10	0	100	0.2

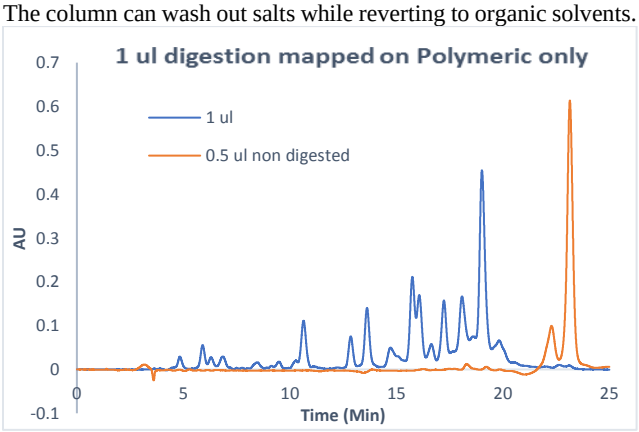
6-In the final step the line and the reversed phase column are flushed clean from any leftover organic going to the enzyme column, still avoiding the Silica column. The setup therefore remains as setup 2.

Time	% of digestion buffer C	Flow rate (ml/min)
0		0
0.01	100	0.3
6	100	0.3

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 digestion is complete, it is reproducible and there is no sign of deactivation of the column's immobilized Trypsin.



Understanding the state of the Mass Spectroscopy, the high-end instruments are now capable of deconvoluting non resolved peaks and high-resolution silica might not be as crucial.
In the chromatogram below, the silica column was avoided, and the separation was run on the polymeric column only.
The use of such polymeric remains apparent especially when high pH solvents with salt are required for digestions.
The column can wash out salts while reverting to organic solvents.



The enzyme column retains its full enzymatic activity after being exposed to large amounts of Trypsin itself.

A note of caution is necessary to keep in mind as the enzyme sample can be subject to auto digestion during an extended period. As the industry moves to greener environment and the use of smaller bore columns becomes necessary, one might keep in mind that background noise is no longer drowned under large amounts of samples. Nor are the solvent drifts during gradient runs. It would become necessary to address it by simply running a blank and deducting it from the run itself. It not only addresses the issue of background drift it also and most importantly prevents the researcher avoiding the unnecessary overly expense of super pure LC solvents.

Inc.