

## APPLICATION NOTE

### Automated Digestion of Trypsin with StyrosZyme® TPCCK-Trypsin, Immobilized Enzyme on Polymeric Hard Gel Simulated-Monolith™ Enzyme Reactor with the Acquity UPLC I class Plus and Final Silica mapping. Result of excess injection of Trypsin on the Trypsin column.

We have shown so far in Application Note 142 that the enzyme column remains stable and is not affected during the digestion of a Trypsin solution. That is would be a controlled auto-digestion.

During this Application Note we propose to increase 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 H<sub>2</sub>O: ACN 98:2 (for peptide mapping)

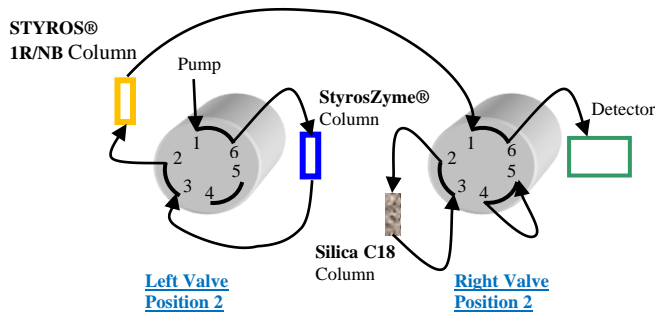
Buffer B: 0.1 % TFA in ACN: H<sub>2</sub>O, 70:30 (for peptide mapping)

Buffer C: 0.1 M Tris, pH= 8.55 (for digestion).

The 3 columns used are also the same:

A Narrow Bore enzyme column of 2.1x50 mm stainless Steel (StyrosZyme® TPCCK-Trypsin), with the same size reversed phase polymeric Narrow Bore column (STYROS® 1R) ending with the Silica C18 column (Acquity UPLC® BEH C18 1.7 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.

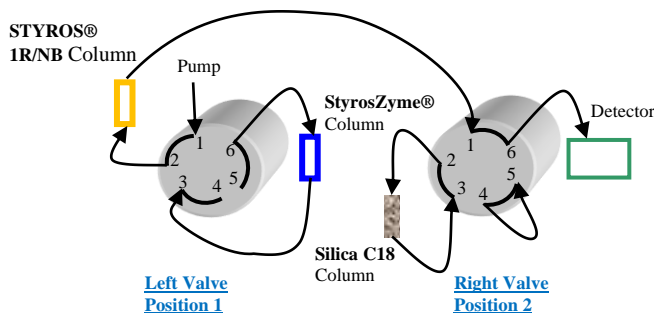
#### 1-Equilibrate the enzyme column with all columns except the silica column, in line, as shown in Setup 1.

| Time | % of buffer C | Flow rate (ml/min) |
|------|---------------|--------------------|
| 0    |               | 0                  |
| 0.01 | 100           | 0.2                |
| 6    | 100           | 0.2                |

#### 2-With both columns in line as in Setup 1, 5 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                  |
| 0.01 | 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                  |
| 0.01 | 0             | 100           | 0.3                |
| 10   | 0             | 100           | 0.3                |

#### 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                |
| 20   | 60            | 40            | 0.2                |

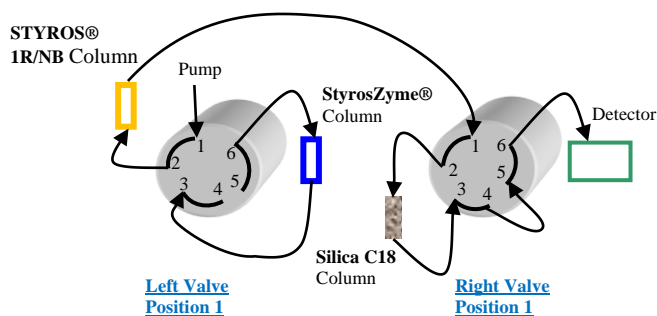
Once the conditions of the digestion are satisfactory, one can use the second switching valve to bring the Silica C18 column online as shown in Setup 3.

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                |
| 20   | 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            | 0.3                |
| 2    | 100           | 0             | 0.3                |
| 4    | 0             | 100           | 0.3                |
| 8    | 0             | 100           | 0.3                |

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           | 0.3              |
| 10   | 0             | 100           | 0.3              |

**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                |

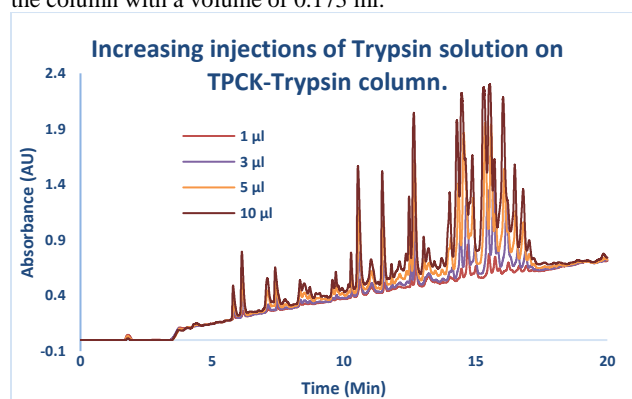
The system is now ready for the next cycle to check the reproducibility of the digestion.

We have used 5 µl of a solution of 10 mg/ml of Trypsin from bovine as sample to digest.

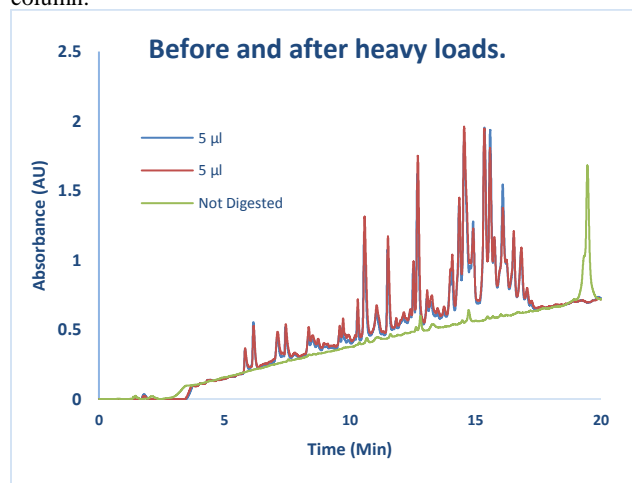
The temperature is set at 37°C for all sequences.

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

Up to 10µl or a solution of 10 mg/ml of Trypsin were injected on the column with a volume of 0.173 ml.



No change is noticed before and after the excess load to the column.



The enzyme column retains its full enzymatic activity after being exposed to large amount of Trypsin itself. The digestion is complete.

A note of caution is necessary to keep in mind as the enzyme sample can be subject to auto digestion during an extended period.

