OraChrom, Inc.
The Vanguard of Liquid Chromatography.

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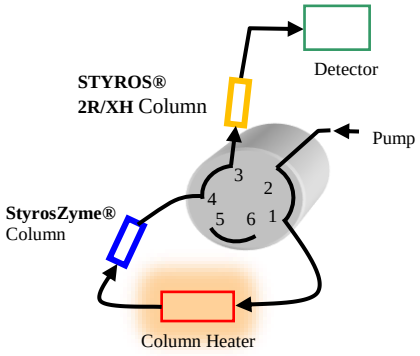
APPLICATION NOTE

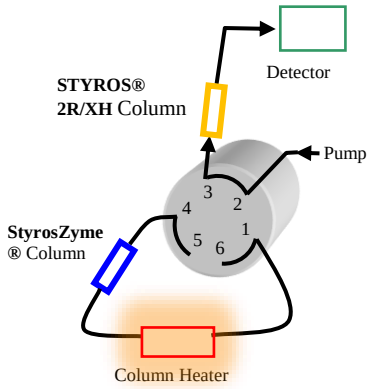
Automated Digestion with StyrosZyme® TPCK-Trypsin, Immobilized Enzyme on Polymeric Hard Gel Simulated-Monolith™.
Digestion and mapping of 3 µg of protein on Narrow Bore column.

Using the Agilent 1290 Infinity as a UHPLC the automated digestion can be run on Narrow Bore columns extending the number of runs with limited amount of solvent as well as minimum amount of sample.
The initial setting of the instrument as depicted below has both columns in series and on line:
During this stage the protein is digested and trapped on the polymeric reversed phase column.



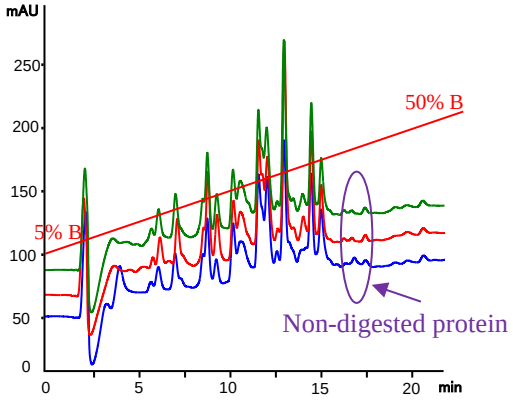
Setup 1

The second setting has only the reversed phase column for the mapping of the resulting peptides.



Setup 2

The digestion and mapping proceeds in under an hour.
It is run at volumetric flow rates of 0.2, 0.4 and 0.8 ml/min. That is from 350 cm/hr to 1,400 cm/hr.
Cytochrome c is digested similarly at all speed interacting with the tethered enzyme for less than a second.
The protein is not treated for digestion and it is in its native form.
Once digested the resulting peptides are trapped on the STRYROS® 2R polymeric reversed phase column to be desalted, mapped and ultimately hyphenated with a mass spectrometer.



A 2.1x50 mm Narrow Bore enzyme columns (TPCK-Trypsin) is used for the digestion followed by a 2.1x150 mm (STYROS® 2R) also Narrow Bore reversed phase column to trap and map the digested peptides.
As a polymeric reversed phase Simulated-Monolith™ column, it can withstand extremes in pHs and has low pressure drops.
The 3 buffers needed are as follows:
Buffer A: 0.075 % TFA in DI H2O (for peptide mapping)
Buffer B: 0.075 % TFA in Acetonitrile: H2O, 95:5(for peptide mapping)
Buffer C: 0.1 M TRIS, pH= 8.5 (for digestion)
The temperature is set at 37°C for all sequences.
A sequence consisting of 6 steps is used in the following order:

1-Equilibrate the enzyme column with both columns in line as shown in Setup 1.

Time	% of buffer C	Flow rate (ml/min)
0		0
0.01	100	0.6
3.00	100	0.6

2-With both columns in line as in Setup 1, 1 µl of a solution of 3 mg/ml of Cytochrome c in buffer A is injected and the resulting digests are dumped on the reversed phase column using the following sequence:

Time	% of buffer C	Flow rate (ml/min)
0	100	0
0.01	100	0.4
8	100	0.4

3- The enzyme column is removed as shown in Setup 2 to wash off the salt and prepare the reversed phase column for mapping. It is also ready for the hyphenation with a mass spectrometer.

Time	% of buffer B	% of buffer A	Flow rate (ml/min)
0			0
0.01	5	95	0.5
10	5	95	0.5
10.1	5	95	0.2
12	5	95	0.2

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

Time	% of buffer B	% of buffer A	Flow rate (ml/min)
0	5	95	0.2
22	50	50	0.2

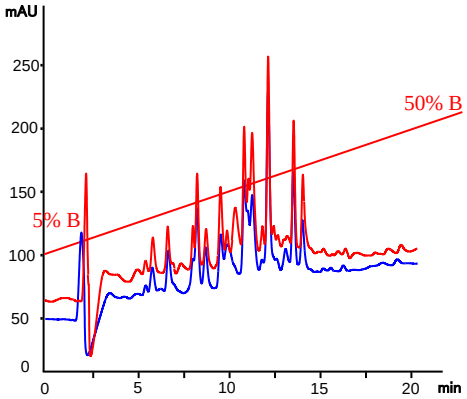
5- The reversed phase column is washed in this step to remove any leftover peptides from the previous digestion. The setup remains as Setup 2.

Time	% of buffer B	% of buffer A	Flow rate ml/min
0	5	95	0
0.01	45	55	0.5
0.5	60	40	0.5
1	60	40	0.5
1.5	45	55	0.5
1.6	5	95	0.5
2.5	5	95	0.5

6-In the final step the reversed phase column is pre-equilibrated with the digestion buffer to wash off all organics from the line.
The setup remains as Setup 2.

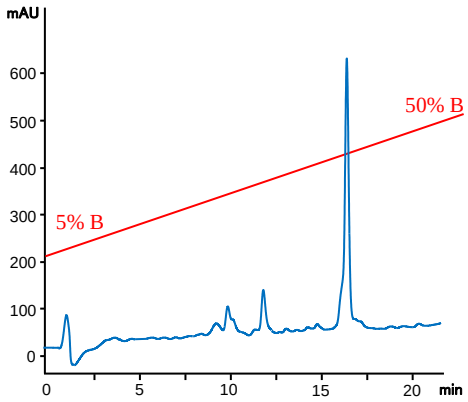
Time	% of buffer C	Flow rate (ml/min)
0		0
0.01	100	0.5
3.5	100	0.5

The pressure drop of the system does not exceed 300 bars even at 0.6 ml/min with both columns on line.
It is therefore safe to run multiple injections overnight with no concern of the system exceeding its pressure limit of 1300 bar at such flow rates.
The digestion remains the same with the length of the enzyme column doubled:

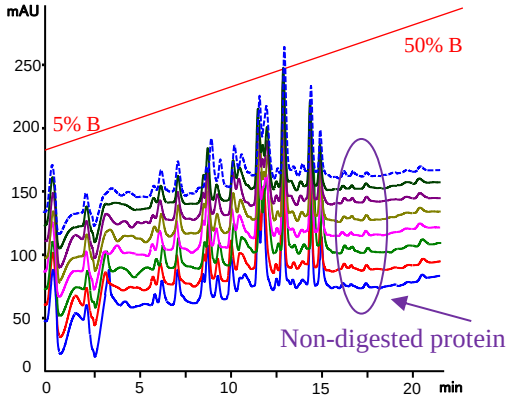


It is also important to run the proteinaceous sample without the enzyme column on line to assess all components that will contribute to the mapping of the resulting peptides.

In the present case the initial Cytochrome c from equine heart shows additional compounds. The sample is assessed over 95% pure by SDS Page by the manufacturer.



To conclude, several digestion and mapping were run using the same setup to check the reproducibility.



There is no change in the amount of non-digested protein or the resulting peptide after more than 200 runs.
The pressure drop remains the same for both the enzyme column as well as for the reversed phase column.

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