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APPLICATION NOTE

Automated digestion of Cytochrome c from equine with StyrosZyme® TPCK-Trypsin, Immobilized Enzyme on Polymeric Hard Gel Simulated-Monolith™ Enzyme Reactor. Comparison of binary with quaternary UPLC systems.

Trypsin is a medium size globular pancreatic serine protease that cleaves protein bounds on the C terminal of the amino acid residues lysine and arginine.
The optimum pH for Trypsin is 9 and the optimum temperature is 65°C. However, these numbers hold true when the digestion proceeds in batch.

In the case of online digestion, we were able to achieve full digestion of the protein at 37°C and pH of 8.5 using 0.1 M Tris buffer.

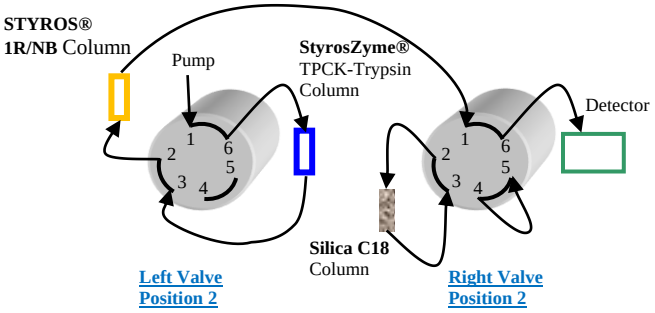
The tandem reversed phase column in such setup needs to tolerate high pH's.
Polymeric reversed phase columns such as STYROS® 1**R and 2R** are highly recommended, especially when the eluents are used to feed a mass spectrometer.

The 3 columns used are all narrow bore, therefore minimizing high solvent consumption as well as the generation of excess waste.
The Enzyme column used is a 2.1x50 mm stainless Steel (StyrosZyme® TPCK-Trypsin).
Two narrow bore reversed phase columns. One polymeric (STYROS® 1R) with high capacity, high pH tolerance and high performance (2.1x50 mm) column as well as a Silica C18 column (ZORBAX Eclipse C18 1.8 µm 2.1x50 cm column) with high performance.

The buffers used are:
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= 8.5(for digestion).

The Waters UPLC *I* class Plus binary system fitted with 2 six port valves was used.

The following shows the starting positions of the 2, 6 port valves used:



Setup 1

In this first position, the StyrosZyme® TPCK-Trypsin column and the polymeric STYROS® 1R/NB column only, are online and the Silica C18 column is not exposed to any excessive pH 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, StyrosZyme® TPCK-Trypsin and STYROS® R reversed phase polymeric can tolerate high pH.

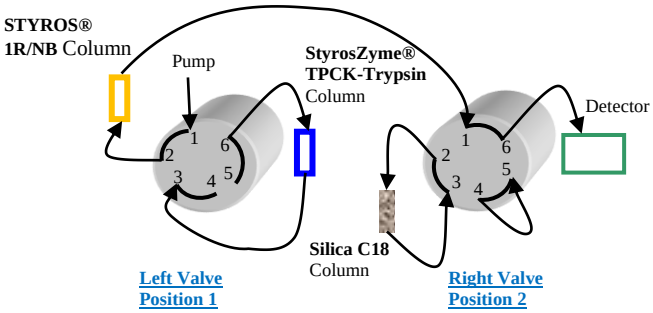
1-Equilibrate the enzyme column with all columns except the silica column, in line, as shown in Setup 1.

Time	% of buffer C	Flow rate (ml/min)
0		0
0.01	100	0.2
8	100	0.2

2-With both columns in line as in Setup 1, 3 µl of a solution of 4.5 mg/ml Cytochrome c Equine in buffer A is injected, and the resulting digests are dumped on the reversed phase polymeric column using the following sequence:

Time	% of buffer C	Flow rate (ml/min)
0		0
0.01	100	0.1
2	100	0.2
5	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 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	5	95	0
0.01	5	95	0.3
9.5	5	95	0.3
9.6	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.

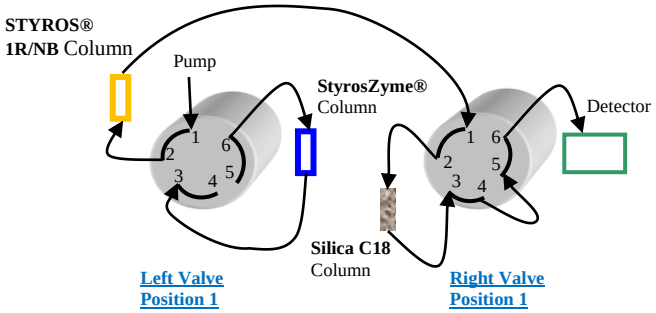
Time	% of buffer B	% of buffer A	Flow rate (ml/min)
0	5	95	0.2
10	80	20	0.2

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:
The Silica C18 column is now a (ZORBAX Eclipse C18 1.8 µm 2.1x50 cm column) from Agilent.
It has a clear different retention than the Waters Acquity C18 column and requires a different gradient as noted above.

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	5	95	0.2
10	80	20	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	5	95	0
0.01	60	40	0.3
2	100	0	0.3
4	5	95	0.3
10	5	95	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	5	95	0.3
10	5	95	0.3

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	0.3
6	100	0.3

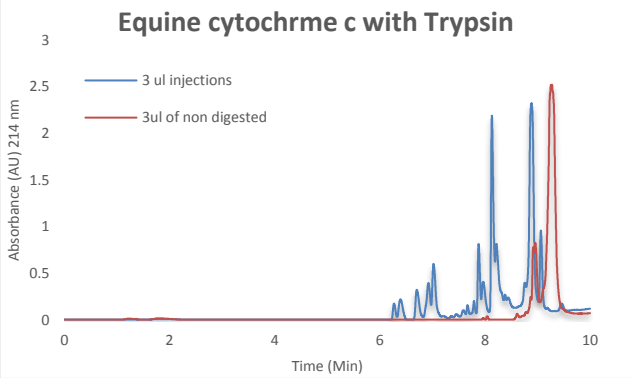
The system is now ready for the next cycle to check the reproducibility of the digestion.

We have used 3 µl of a solution of 4.5 mg/ml of protein in buffer A as sample to digest.

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

The digestions are done under similar conditions. Although temperatures as high as 60°C and as low as 10°C have been used with full digestion, and flow rates of 0.4 ml/min, that is a linear flow rate of 693 cm/hr for an empty column of 2.1 mm ID, were also experimented with, with the same result of full digestion.
The enzyme column of 2.1 x 50 mm has a volume to 0.173 ml. A total of 1 ml is run through it during the digestion at a volumetric flow rate of 0.1 to 0.2 ml/min.

The performance of the STYROS® 1R polymeric is crucial in the final separation of the digested peptides,

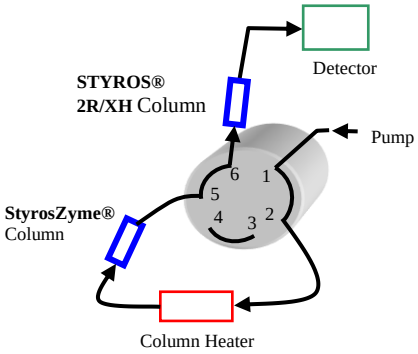


Same amount of protein (3 µl) is injected under the same conditions.
In the setup 3, during a 10-minute gradient run, the trapped peptides are mapped on both the polymeric and the C18 Silica column.
The amount injected is clearly saturating the narrow bore polymeric and silica columns. This is the amount of protein that is run through the column and shows full digestion.

This process has been compared to the digestion of the same amount of protein on an Agilent 1260 Infinity *I* quaternary pump with minimal variation.
No silica column was used as the new mass spectroscopy instruments can readily handle separations. Only if the end user is limited to LC instruments and relies on it for his separations, then the use of an additional silica column would be warranted.
To be simple, we have used two polymeric columns of 2.1 x100 mm. One StyrosZyme TPCK-Trypsin® and one STYROS® R1 reversed phase polymeric of the same size.

1-Equilibrate the enzyme column and the reversed phase polymeric column as shown in Setup 1 using a single 6 port valve in positions 1 to 2.

Time	% of buffer C	Flow rate (ml/min)
0		0.2
0.01	100	0.3
6	100	0.3

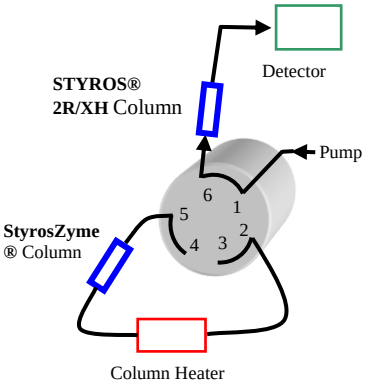


Setup 1

2-With both columns in line as in Setup 1, 3 µl of a solution of 4.5 mg/ml Cytochrome c Equine in buffer A is injected, and the resulting digests are dumped on the reversed phase polymeric column using the following sequence:

Time	% of buffer C	Flow rate (ml/min)
0		0.2
0.01	100	0.2
10	100	0.1

In the second setup, the valve is switched in positions 1 to 6 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		100	0.2
25	85	15	0.2

5-In this step, using the same setup 2, the polymeric column is washed with high organic to make sure it is devoid of any leftover proteinaceous species.

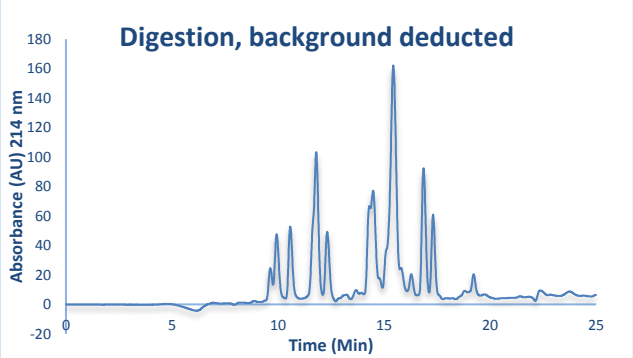
Time	% of buffer B	% of buffer A	Flow rate (ml/min)
0	60	40	0
0.01	60	40	0.3
1	100	0	0.3
4	0	100	0.3
8	0	100	0.3

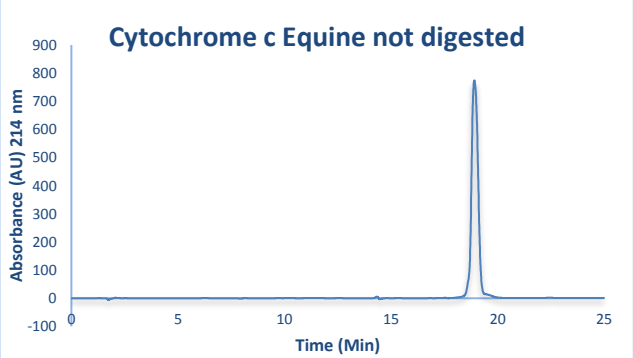
6- The reversed phase columns is preequilibrated to the initial low organic prior to getting in contact with the digestion buffer.
The setup is now Setup 1.

Time	% of buffer B	% of buffer A	Flow rate ml/min
0			0
1	0	100	0.3
10	0	100	0.3

The following chromatograms are a good indication that the present mass spectroscopy instruments can be satisfied with such separations and no additional fine separation is needed.

In addition to make it one step simpler, it also allows most digested species that can hydrophobically be trapped to the reversed phase polymeric be accounted for.
The next step would be to eliminate the reversed phase column and dump the result of the digestion into the mass at the expense of additional cleaning of the mass chambre.





As the above chromatogram shows the digestion is complete in less than 10 minutes under mild conditions without the need for denaturing and alkylation that is burdensome.
The presence of auto digest is another benefit of online digestion as well as the leaching of the media used for immobilization of the enzeymes.
The presented process prevents the autodigestion of the enzyme that plagues the batch digestion in addition to the anachronistic time required for its completion.

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