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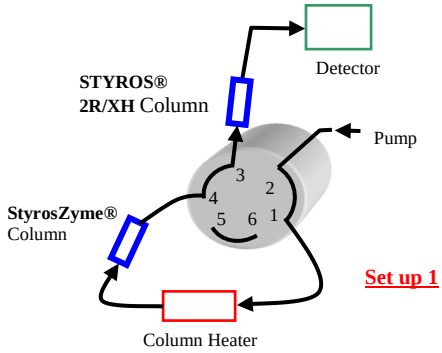
APPLICATION NOTE

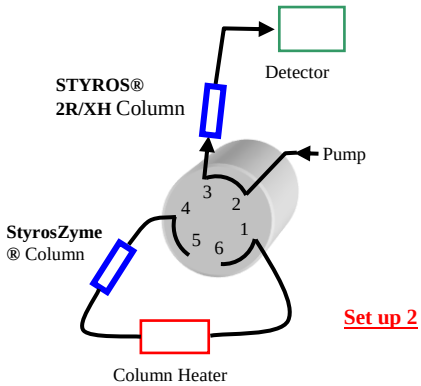
StyrosZyme® TPCK-Trypsin, Immobilized Enzyme on Polymeric Hard Gel Simulated-Monolith™. Automation of the Digestion and Mapping of Cytochrome c.

Digestion of proteins or proteinaceous entities is a tedious process that is not reproducible and tainted with auto digests of enzymes used in the process.
Immobilized enzymes however have made it possible to avoid this and when immobilized on stable polymeric media they have paved the way to automation.
The present application note describes the details of the automated digestion of Cytochrome c from equine heart using a basic HPLC instrument (HP 1100 with quaternary pump, now Agilent Technologies) and the OpenLab Software.
Using a 6-port valve the enzyme column along with the polymeric reversed phase STYROS® 2R are connected as depicted in these following schematic diagrams:
In the first position the enzyme reactor (StyroZyme® TPCK-Trypsin) is connected in series with the STYROS® 2R column.



Set up 1

In the second position the enzyme column is removed from the line and only the reversed phase STYROS® 2R column remains.



Set up 2

The 3 buffers needed are as follow:

Buffer A: 0.1 % Formic acid in DI H2O (for peptide mapping)
Buffer B: 0.1 % Formic acid in Acetonitrile: H2O, 95:5(for peptide mapping)
Buffer C: 0.1 M TRIS, pH= 8.5 (for protein digestion)

The temperature on the column heater is set at 37°C for all sequences.
The enzyme column is a 2.1x50 mm stainless Steel column (StyrosZyme® TPCK-Trypsin).
The reversed phase column is a 4.6x150 mm STYROS® 2R.
As a polymeric reversed phase Simulated-Monolith™ it can withstand extremes of pH's and has low pressure drop.
A sequence made of 6 steps is used in the following order with a total time of less than 40 minutes.

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

Time	% of buffer C	Flow rate (ml/min)
0		0
0.01	100	1
4	100	1
4.1		0

2-With both columns in line as in set up 1, 10 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		0
0.01	100	0.1
2	100	0.5

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

Time	% of buffer B	% of buffer A	Flow rate (ml/min)
0			0
0.01	5	95	1.5
5	5	95	1.5
5.1			0

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

Time	% of buffer B	% of buffer A	Flow rate (ml/min)
0			0
0.01	5	95	1
16.6	35	65	1
17			0

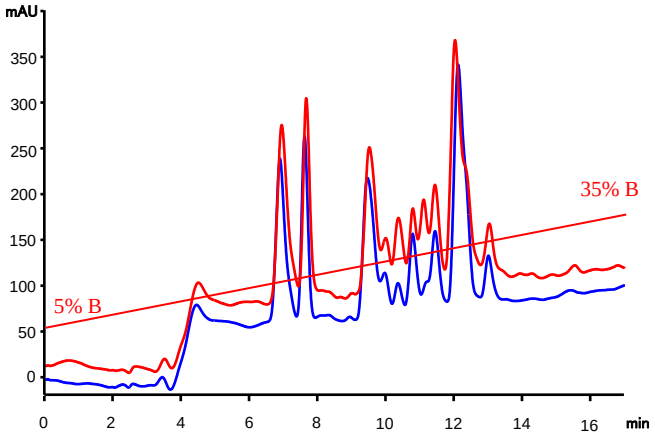
5- The reversed phase column is washed in this step to remove any leftover from the previous digestion.
The set up remains as set up 2.

Time	% of buffer B	% of buffer A	Flow rate ml/min
0			0
0.01	45	55	1
1	80	20	1
2	90	10	1
4	90	10	1
5	5	95	1
7	5	95	1

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 set up remains as set up 2.

Time	% of buffer C	Flow rate (ml/min)
0		0
0.01	100	0.5
4	100	0.5
4.1		0

The result of the digestion and mapping of the same immobilized enzyme (TPCK-Trypsin) in columns of different diameters (2.1 and 4.6 mm) is shown here compared to one another.
The primary purpose of this Application Note is to show the feasibility of the digestion of proteins to be fully automated.
The digestion is complete and reproducible.
The enzyme reactor has a long shelf life as it is the case with most non-leaching immobilized enzymes.



Chromatogram 1
Mapped digestion of Cytochrome c with StyroZyme® TPCK-Trypsin Simulated-Monolith™ (4.6x50 mm and 2.1x100 mm)

Operating parameters.

HPLC System.	Agilent 1100 with thermostatted column compartment and a 6-port valve.
Columns	**StyrosZyme® TPCK-Trypsin 2.1 X 100 mm/** **StyrosZyme® TPCK-Trypsin** 4.6 X 50 mm **STYROS® 2R/XH** 4.6 X 150 mm
Mobile phase.	A: 0.1 % Formic Acid B: 0.1 % Formic acid in Acetonitrile: H2O 95:5 C: 0.1 M Tris, pH=8.5
Flow rates	As indicated in various steps
Gradient	As indicated in various steps
Temperature	37°C
Detection	214 nm
Injection volumes	10 µl
Sample:	3 mg/ml of Cytochrome c in buffer A.

The reversed phase column used in this Application Note is a Simulate-Monolith™ polymeric. Therefore, the concern about the pore size no longer applies. Pores are through-pores and can accommodate molecules of all sizes.
The capacity, retentiveness and chemical tolerance of the media is high enough to be used as a trap for the digests as well as for their desalting followed by mapping.
It can tolerate extremes of pH used for the digestion.
Should the process be used with the LC as the end result, the separation can be improved using a shallower gradient and different additive such as TFA.

It would be appropriate and important to explore:

- The length of the enzyme column and its effect on the digestion,
- The amount of substrate and its effect on the digestion,
- Increasing the interaction time between the substrate and the enzyme by reducing the flow rate during digestion.
- Increasing the ID of the enzyme column and therefore modifying the linear velocity during digestion.
- Decreasing the temperature and running the digestion at room temperature of 20°C.
- The optimum linear velocity for the digestion in a given setting?

These are the questions explored in Application Note 133.

Inc.